

2013

Development Of Micro Volume Dna And Rna Profiling Assays To Identify The Donor And Tissue Source Of Origin Of Trace Forensic Biological Evidence

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DEVELOPMENT OF MICRO VOLUME DNA AND RNA PROFILING ASSAYS TO
IDENTIFY THE DONOR AND TISSUE SOURCE OF ORIGIN OF TRACE FORENSIC
BIOLOGICAL EVIDENCE

by

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A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Forensic Science
in the Department of Chemistry
in the College of Sciences
at the University of Central Florida
Orlando, Florida

Fall Term
2013

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ABSTRACT

In forensic casework analysis it is necessary to obtain genetic profiles from increasingly smaller amounts of biological material left behind by perpetrators of crime. The ability to obtain profiles from trace biological evidence is demonstrated with so-called ‘touch DNA evidence’ which is perceived to be the result of DNA obtained from shed skin cells transferred from donor to an object or person during physical contact. However, the current method of recovery of trace DNA involves cotton swabs or adhesive tape to sample an area of interest. This “blind-swabbing” approach may result in the recovery of biological material from different individuals resulting in admixed DNA profiles which are often difficult to interpret.

Profiles recovered from these samples are reported to be from shed skin cells with no biological basis for that determination. A specialized approach for the isolation of single or few cells from ‘touch DNA evidence’ is necessary to improve the analysis and interpretation of recovered profiles. Here we describe the development of optimized and robust micro volume PCR reactions (1-5 μ L) to improve the sensitivity and efficiency of ‘touch DNA’ analysis. These methods will permit not only the recovery of the genetic profile of the donor of the biological material, but permit an identification of the tissue source of origin using mRNA profiling.

Results showed that the 3.5 μ L amplification volume, a fraction of the standard 25 μ L amplification volume, was the most ideal volume for the DNA assay, as it had very minimal evaporation with a 50% profile recovery rate at a single cell equivalent input (~5 pg) with reducing amplification volume alone. Findings for RNA showed that by reducing both amplification steps, reverse transcriptase PCR (20 μ L) and body fluid multiplex PCR (25 μ L), to

5 uL, ideal results were obtained with an increase in sensitivity and detection of six different body fluids down to 50 pg.

Once optimized at the trace level, the assays were applied to the collection of single and few cells. DNA findings showed that about 40% of a full profile could be recovered from a single buccal cell, with nearly 80% of a full profile recovered from only two cells. RNA findings from collected skin particles of “touched” surfaces showed accurate skin detection down to 25 particles and detection in one clump of particles. The profiles recovered were of high quality and similar results were able to be replicated through subsequent experiments.

More studies are currently underway to optimize these developed assays to increase profile recovery at the single cell level. Methods of doing so include comparing different locations on touched surfaces for highest bio-particle recovery and the development of physical characteristics of bio-particles that would provide the most ideal results.

ACKNOWLEDGMENTS

I would first like to thank Dr. Jack Ballantyne for allowing me the opportunity to be a part of his laboratory and learn more than I ever thought was possible. Without this opportunity, I would not have discovered what I truly want to make a career out of and direct my life towards. It has been an honor to work as a part of your laboratory. I want to thank Dr. Erin Hanson for her patience with me and the knowledge she has passed onto me. She has opened me up to thinking beyond what is in front of me, and although I am still learning, I will take these skills with me wherever I go. I would also like to thank my committee members, Dr. Dmitry Kolpashchikov and Dr. Jingdong Ye, for taking the time to time to be a part of this process. Finally, I would like to acknowledge the other laboratory members who have helped me along the way and those who have donated body fluids in aid towards my research.

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LIST OF ACRONYMS (or) ABBREVIATIONS

Amp	Amplification
cDNA	Complementary DNA
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxynucleotide Triphosphates
LCN	Low copy number
mIPEP	Modified improved primer extension preamplification
MP	Multiplex
mRNA	Messenger ribonucleic acid
NCFS	National Center for Forensic Science
PCR	Polymerase chain reaction
PEP	Primer extension preamplification
RFU	Relative fluorescent unit
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SA	Saliva
STR	Short Tandem Repeat
VS	Vaginal

CHAPTER ONE: INTRODUCTION

When a crime occurs, biological tissue or fluid is usually the most sought after collectable evidence. Whether belonging to the victim or the suspect, this biological evidence can be the most important part of a criminal case. For this reason, development of highly successful identification methods through biological evidence has been the forefront of the forensic field [1, 2]. Analysis of deoxyribonucleic acid (DNA) is still relatively new, really getting its roots in 1985 by researcher Alec Jeffreys. He noticed that certain regions of DNA tended to repeat randomly, and the amount of times those regions repeated, for the most part, were unique to each individual. Jeffreys found that by using restriction enzymes, enzymes that cut DNA strands at dictated locations, he could isolate these sections of repeated DNA and compare them between individuals. The name given to this technique was restriction fragment length polymorphism (RFLP). His discovery changed the way that forensic biological samples were analyzed and started the concept of “DNA fingerprinting” [1-4]. Over time, RFLPs were phased out by the study of the more accurate short tandem repeats (STRs) [6-11]. In combination with capillary electrophoresis, STR typing can produce highly discriminating profiles [7].

It is also of interest to determine origin of the biological sample. The context of the crime could entirely depend on the type of body fluid recovered (i.e. blood, semen, saliva, menstrual blood, skin, and vaginal secretions). Current serological testing can be very laborious, time consuming, requires a large amount of sample, and aren't generally very specific. A new direction has been brought forth to alleviate the issues towards the analysis of messenger ribonucleic acid (mRNA) [12]. The mRNA is the intermediate from DNA to proteins. The

mRNA molecule is transcribed, or copied from, DNA to eventually be translated into amino acids. These amino acids will then be formed into proteins that are specific to that tissue type [12-18]. Different studies have looked into different body fluid specific genes, but the most current targeted genes as discovered and validated by the National Center for Forensic Science are shown in Table 1 (TGM4 and LEFTY2 unpublished by NCFS).

Table 1: Current body fluid genes utilized by NCFS

Body Fluid	Body Fluid Specific Gene	Function
Blood	ANK1 ¹⁹	Binds to Membrane Proteins to Maintain Structure of RBC
	ALAS2 ²⁰	Heme Synthesis
Semen	PRM2 ^{13,14,15,16,20,21, 22}	Compact Sperm Chromatin
	TGM4 ^{16,19}	Catalyzes cross-linking of protein chains (specific to seminal tract)
Saliva	HTN3 ^{12, 13,15,16,20,21}	Histatin, oral cavity defense
	STATH ^{12, 13,115,16,20,21}	Inhibits calcium phosphate salt precipitation (oral cavity)
Vaginal Secretions	CYP2B7P1 ²³	Psuedogene
Menstrual Blood	MMP10 ²⁰	Degrades cell membrane Activates procollagenase
	LEFTY2 ²⁴	Asymmetry determination
Skin	LCE1C ^{25,26}	Development of Stratum Corneum
	CCL27 ^{25,26}	Mediates lymphocyte homing to cutaneous locations

Today, the need to obtain identifying profiles from increasingly smaller amounts of biological evidence is becoming more common. These samples are known as trace evidence. Trace evidence is occasionally called “touch” evidence, implying that direct contact was made by skin, and cells shed onto the surface. This isn’t always the case, however, as trace evidence can include many different body tissue types, just not in a visible form as these samples are very minute [27]. Due to the growing interest in the analysis of trace samples, the possibilities of biological evidence collection has vastly expanded, with numerous studies demonstrating the ability to obtain identification from trace evidence from many different surfaces such as shoe insoles [28], touched documents [29], bedding [30], car interiors [31] and even on the victim, such as the contact during manual strangulation [32]. Trace samples are usually collected in a variety of ways. “Blind swabbing”, or vigorous swabbing of assumed contacted surfaces with either a wet or dry swab, is the most common form of collection [33]. Several studies have suggested the best collection method to be that of multiple swabbing due to biological material left behind from the first swab [34, 35]. Another form of trace evidence collection is the use of tape lifts [36]. While these methods are convenient, they pose major problems that usually affect analysis downstream. When blind swabbing, unintentional pick up of cells from multiple donor sources can occur. Due to natural difficulty of mixture analysis, when samples already start with low inputs, separation of individual profiles may be impossible [37]. Even if a mixture isn’t created through swabbing, other particles and debris can be picked up in the swab/tape, which can cause inhibition issues when biological input is already low, and with standard analysis methods, a full quality profile can be difficult to obtain.

Another option has been studied as a possible solution to these problems: the application of low copy number (LCN) analysis techniques to the analysis of trace samples. LCN samples typically have around 100 pg or less of biological input (1 ng is standard, with a single cell having about 5 pg). A term often confused with touch, it is important to distinguish the difference between sample types. As previously describe, touch involves contact. As some LCN samples may involve contact, not all do. Other forms of LCN samples include degraded biological materials [38], often due to environmental effects, such as the identification of badly damaged or skeletonized remains after mass disasters [39, 40]. Unfortunately, LCN analysis is still struggling to gain acceptance into the forensic community [41]. In the past, LCN profiles have been riddled with stochastic effects, such as major allelic drop out, ultimately ruling out its admissibility in court [41, 42]. More recent studies, though, have started showing progress towards acceptance, and since trace analysis is admissible, these developed methods have been applied to increase the ability of obtaining higher quality profiles from trace samples.

One method that has been brought up in numerous past studies is whole genome amplification [44-49]. Hanson and Ballantyne [48] focused their study on few cell and single cell equivalents and worked to develop an optimal protocol best suited for analysis of these trace samples. In their study, a modified version of primer extension preamplification (PEP) was developed, titled mIPEP. In order to develop this new technique, the PCR conditions were first examined. In previous clinical genetics studies, it was reported that by increasing several components, such as the amount of primer used, there was higher genotyping success with low DNA quantities [49, 50]. The original PEP method was also examined and several modifications were made that included increasing the denaturation temperature and taking out an additional

elongation step from each PCR cycle [48]. By applying this newly developed method, sensitivity was dramatically increased allowing for full profiles down to 5 pg of DNA, a single cell equivalent, (as a modification, a reaction volume of 12.5 μ L was used with 32 cycles for amplification). Furthermore, two types of polymerase was used [48], which allowed for more “proof-reading” and sensitivity was increased further by raising the amount of enzyme mix used [48-50]. Several other methods of whole genome amplification, such as multiple displacement amplification and degenerate oligonucleotide-primed PCR, were evaluated in the study but were each unsuccessful in obtaining profiles from single cell DNA equivalent levels, as mIPEP was successful with. The issue with this method is its application to the forensic field. Whole genome application is very time consuming. While this may be acceptable in clinical fields, forensics is highly dependent on time. In addition to time, this method requires extra steps beyond standard analysis which requires specialized training. This would not be well suited to forensic casework.

Another possible option for trace biological analysis is post-amplification purification [51, 52]. Unlike whole genome amplification, which aims to increase the quality of sample before STR amplification, post-amplification purification aims to increase the quality of the samples for capillary electrophoresis. During this process, due to an injected electrode, DNA molecules are drawn into the capillary. Due to the increase in resistance as the voltage is applied, smaller components of the amplified sample, such as dNTPs and salts, are favored to be pulled up over DNA. By taking out these smaller components, through post-amplification purification, there should be nothing inhibiting the DNA molecule to be pulled up [51, 52]. Smith and Ballantyne [51] intended to apply this method to the analysis of trace samples. The main aim of their study was to attempt to increase the fluorescent allelic strength by utilizing post-

amplification purification to increase sensitivity without extra steps beyond purification. Four techniques of two purification methods were applied; for filtration there was Microcon-50 and Montage PCR, silica columns included Qiagen MinElute, and enzyme-mediated hydrolysis ExoSAP-IT. The profiles obtained were compared by the quality of the profile and the RFU values (relative fluorescent units). They found that the Montage method provided the greatest increase in RFU values when all techniques were compared at 156 pg, 78 pg, 39 pg, and 20 pg. ExoSAP-IT was excluded due to poor quality data, such as low RFU values and split peaks. For single cell equivalents that were purified by MinElute, full profiles were obtained down to 78 pg and partial profiles at about 5-10pg. For non-purified samples, full profiles were obtained at 156 pg but were weak in signal. Partial profiles were obtained down to 39 pg. Within their study, profiles did show an increase in stutter, allelic drop in, and heterozygote peak imbalance [51]. Although this method has shown promise towards better trace analysis, it still requires extra time, materials, and training. Another method has been studied, as described below, that doesn't require extra time or training and can even save material and sample.

Reduced volume amplification is another possible method that is steadily gaining popularity [53, 54]. The idea behind reduced volume when applied to trace samples is that by reducing what extra reagents go into the amplification mix, the primers face less inhibition when attaching to the individual strands of DNA, allowing for greater efficiency and sensitivity. More benefits, which is ideal when applied to crime laboratories, is that there is no extra time added to analysis, it follows the natural flow of the standard procedures so no extra training is required, and it cuts costs by reducing reagent use and saves samples for further testing. Gaines, et al. [54] looked to examine the true potential of these benefits and aimed to address a concern of the

method that the kinetics would be altered due to possible evaporation of sample. Setting out a list of five experiments, the first involved reducing the amplification volume proportionally with lowering the amount of DNA input. The second used the same DNA input while lowering the volume. The third experiment evaluated and determined the efficiency of the previous experiments. The fourth aimed to find the minimum detection volume that would provide quality results. Last, mixture samples were tested with the reduced volume method developed through the previous experiments. The authors found that despite the DNA input amount (2 ng down to 200 pg) and reaction volume (25 μ L down to 5 μ L, with 10 μ L and 5 μ L covered with mineral oil to prevent evaporation) the results of quality of the profiles were very similar. However, the lower DNA inputs tended to show an increase in heterozygote imbalance. It was also found that when only the reaction volume changed, the PCR product remained the same for the 15 μ L, 10 μ L, and 5 μ L but the lower volumes showed quality limitations when the DNA input was at 2 ng. Similar results were found with the mixture samples, with increasing interpretation difficulty and increased artifacts as the ratio of major donor and minor donor increased. It was concluded, however, that reduced volume is very useful in increasing the sensitivity successfully but would require more investigation and enhancement [54].

More investigation into reduced volume brought about the development of chemically structured chips in a glass slide format that is directed for micro volume use of 1 μ L amplification volumes [53, 55, 56]. Proff, et al. [53] evaluated the use of the chemically structured chip through a series of experiments. The chip that was used in this study was a 60 well glass microscopic slide. The 60 wells are small hydrophilic circles surrounded by a hydrophobic ring to prevent the PCR reagents and samples from spilling out and mixing with the

other samples. Each sample is covered with a sealing solution to prevent evaporation of the products. In this particular study, several DNA inputs in intervals from 2.5 ng down to 10 pg were evaluated while using standard PCR cycles. Along with sensitivity testing, mixtures and relevant forensic casework samples were investigated as well. Each sample was amplified using three different kits: Blue, SEfiler, and Identifiler (Applied Biosystems). Full profiles were obtained at 40-50 pg for Blue, 80-90 pg for SEfiler, and 150-200 pg for Identifiler, but these profiles showed an increase in heterozygote peak imbalance. Partial profiles were obtained at DNA inputs of 10-20 pg for all three kits used. Peaks at the lower reaction volumes tended to show higher RFU values than those compared to a typical 25 μ L reaction volume except for inputs above 600 pg. These inputs showed more pull up artifacts, but is most likely due to overloading. Mixtures showed successful results up to a ratio of 10:1. When evaluating relevant forensic casework samples, the results were very similar to those at the typical 25 μ L reaction volume levels. At lower DNA inputs, an increase in heterozygote peak imbalance and allelic dropout were observed [53].

While numerous methods have shown success with trace samples, many still have several draw backs that make them inadequate for forensic laboratories. Out of the different studies of trace sample analysis, reducing amplification volume provided the most benefits in line with what is needed in forensics, but current studies still fall short of providing the most ideal results with trace biological samples. With the development of chemically structured chips, they are designed for direct application of collected cells. There has yet to be a study based on removing individual cells or particles from trace biological samples and applying them directly onto the chips. By taking advantage of this ability, time is saved by removing extraction and quantitation

since the user would know exactly how many cells are collected, costs would be cut by utilizing micro volumes, less risk of sample loss due to minimal transportation, and, based on results of previous studies of single and few cell equivalent DNA, utilizing this micro volume would provide highly sensitive results. Aside from DNA, it should be noted that there is an overwhelming lack of concern for body fluid identification. It's a common misconception that when a trace sample is collected from a surface and a DNA profile is obtained; that it indicates that touch occurred through skin contact. While many trace samples are of skin origin, it would be negligent to assume that every sample is of skin origin as context of crime can be determined by body tissue involved. It needs to be made clear that as many skin cells are shed daily but as epithelial cells are pushed to the outer layers of skin, they flatten out and tend to lose their nuclei, classified as particles when deposited onto a surface, in order to form a protective barrier over the body. A profile cannot be obtained if there is no biological material left (Figure 1).

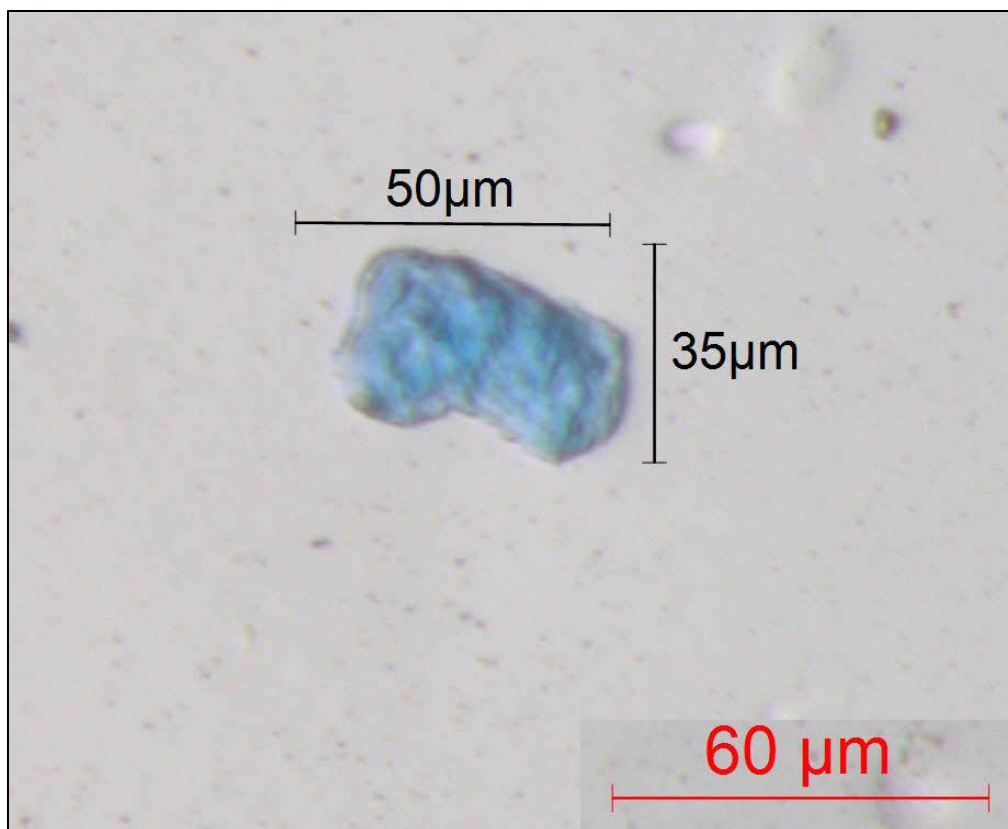


Figure 1: Image of particle(s) collected from coffee mug grip

Problems also arise when mixtures are involved, and it is of utmost importance to match each profile obtained to the correct body tissue. As there are no indicative studies on the matter, a possible solution to these problems could be to also collect single cells or particles to be used for mRNA profiling and adapt analysis to the chemically structured chip. Therefore, there is an overwhelming need for the development of optimal and robust micro volume DNA and RNA assays of trace samples for the adaption of single and few cell analyses.

The intent of this study was to develop the necessary micro volume DNA and RNA assays, first through optimization of trace samples and single cell equivalents, and then to, in combination with a developed cell collection method, apply individual cells directly to a chemically structured chip to obtain identification of individual and body tissue origin. The chemically structured chip instrumentation chosen was the Advalytix AmpliSpeed Slide Cycler. The Advalytix system had been used in the microbiology and physical anthropology fields for the analysis of single cells [57, 58], but hadn't gain widespread use in the forensics field. However, during the beginning stages of development, it was discovered that the Advalytix system had been recalled and would no longer be available for purchase. Although development on the Advalytix system would no longer be an ideal choice, another opportunity was brought forth. The study shifted to the possibility of trace and single cell optimization on standard forensic laboratory equipment that was readily available in most laboratories. If optimization was possible, there would be no need for laboratories to purchase any extra equipment and they would already be trained on the equipment needed. With the direction of this study modified, the new intent was to optimize the DNA and RNA micro volume assays on the standard 9700 thermal cycler. In order to do so, multiple volumes were tested, in intervals from standard

volume down to micro volume levels, while decreasing biological input from standard to trace levels, including single cell equivalents. Once these assays reached what was decided as the optimal micro volume with the most ideal results, collected trace samples would then be analyzed. In order to do so, micromanipulation would be applied. Micromanipulation has been used for numerous years in molecular analysis within immunological studies [59, 60] but hasn't been widely used in forensics yet. It allows greater accuracy of collection over other techniques, such as laser capture microdissection [61-63], but lacks an automatic process. Utilizing a specialized Gel-Film, adhered to a glass slide, the surface of the gel would be pressed against assumed touched items. Due to the properties of the Gel-Film, cells/particles could be easily transferred off of the surface. The Gel-Film could also be stained directly, minimizing potential sample loss due to transport. Under a stereomicroscope, a water soluble adhesive was collect onto the end of a tungsten needle, which will then be able to lift cells/particles off of the surface of the gel. These cells were then placed into the developed micro volume assay (depending on either if for DNA or for RNA analysis) reaction mix and will be continued through analysis. Developing a success method for analysis of single cells, that incorporates micro volume and its benefits, will have the potential to completely eliminate mixtures at the lower trace level. It will also provide a greater prospective on the context of the crime, which may have been previously unknown due to being deemed unnecessary to test. It could also mean less cold cases, or long unsolved cases, as there are more options for biological evidence collection and analysis, and could reduce the risk of sending the innocent to prison, and leaving the criminals in society. This study really has the potential to revolutionize biological analysis and really advance the field of forensic biology.

CHAPTER TWO: METHODOLOGY

DNA Methodology

Sample Collection

Body fluids were collected from numerous volunteers following guidelines set by the university's institutional review board. For blood samples, 50 μ L was collected via venipuncture and was deposited onto sterile pieces of cloth. Semen samples were collected in 50 μ L conical tubes. Swabs were placed into the conical tube and allowed to soak up sample. Saliva samples were collected by using a cotton-tip swab to rub the inside of the cheek.

Extraction

The extraction was carried out automatically via the Qiagen QIAcube instrument with the DNA Investigator Kit. The entire process of extraction was divided into three parts. In the first part, dried samples were placed into individual tubes and placed into the instrument shaker. The final collection tubes and the Qiagen MinElute spin columns were placed into rotor adapters, which are small tube holders for placement into the built in centrifuge. Once the lysis program was started on the instrument, Buffer ATL and proteinase K was added to each sample. Once the

program was complete, the samples were removed from the instrument for the second part of the extraction. Using tweezers cleaned with ethanol wipes, samples were removed from their tubes, placed into spin baskets, and placed back into their tubes. The tubes were centrifuged at 13,200 rpms for three minutes. Samples and spin basket were removed and discarded; the tubes were placed back into the instrument. The third part, the purification program, follows addition of several reagents: Buffer AL and ethanol (200 proof) to complete lysis and prepare conditions for DNA binding, and after the spin columns were placed into the collection tubes: Buffer AW1, Buffer AW2, and more ethanol were added to the column to remove contaminants from bound DNA, and finally Buffer ATE, which was set to 60 μ L, was added to elute the DNA from the column and into the collection tubes. Steps 1 and 3 were carried out entirely by the instrument.

Quantification

The Quantifiler Human DNA Quantification Kit (Applied Biosystems) along with the 7000 Sequence Detection System (Applied Biosystems) was used for real time PCR quantification. The 96-well plates were prepared with 23 μ L of the PCR and primer reaction mix and 2 μ L of either standard or sample. The program conditions were 96°C for 10 min and then 40 cycles of 95°C for 10 sec and 60°C for 1 min. After the program was complete, the results were analyzed and the concentration values were provided for each sample.

Amplification

All DNA samples were amplified using the AmpF ℓ STR $\text{\textcircled{R}}$ Identifiler TM PCR Amplification Kit (Applied Biosystems) with a 95 $^{\circ}\text{C}$ 11 min incubation period which was followed by 28 cycles of 94 $^{\circ}\text{C}$ for 1 min, 59 $^{\circ}\text{C}$ for 1 min, and 72 $^{\circ}\text{C}$ for 1 min, then a 45 minute extension for 60 $^{\circ}\text{C}$ and 4 $^{\circ}\text{C}$ hold. The Identifiler kit contains primers to amplify 15 STR loci and also Amelogenin for sex determination. AmpF ℓ STR $\text{\textcircled{R}}$ Identifiler Plus TM PCR Amplification Kit (Applied Biosystems) with a 95 $^{\circ}\text{C}$ 11 min incubation period which was followed by 29 cycles of 94 $^{\circ}\text{C}$ for 20 sec and 59 $^{\circ}\text{C}$ for 3 min, ending with a 10 min 60 $^{\circ}\text{C}$ extension and 4 $^{\circ}\text{C}$ hold. The Identifiler Plus kit amplifies the same loci as Identifiler, but has been improved with qualities such as enhanced profile quality and efficiency, higher peaks, and has been adapted to work better with difficult samples. Male samples were amplified with AmpF ℓ STR $\text{\textcircled{R}}$ Yfiler TM PCR Amplification Kit (Applied Biosystems) with a 95 $^{\circ}\text{C}$ 11 min incubation period which was followed by 30 cycles of 94 $^{\circ}\text{C}$ for 1 min, 61 $^{\circ}\text{C}$ for 1 min, and 72 $^{\circ}\text{C}$ for 1 min, ending with an 80 min 60 $^{\circ}\text{C}$ extension and 4 $^{\circ}\text{C}$ hold (Applied Biosystems). Yfiler is exclusive to amplify 17 loci on the Y chromosome. This kit is especially useful when determining male profiles when mixed with female tissue, for example in sexual assault situations. All samples were amplified on a GeneAmp PCR 9700 thermal cycler (Applied Biosystems).

Reduced Amplification Volume Experiments

To determine sensitivity levels of the overall amplification volume, the samples were diluted to 1 ng, a standard input used, and were tested with standard volume (25 μ L), 10 μ L, 5 μ L, 2.5 μ L, and 1 μ L. Due to possible evaporation issues with 1 μ L, additional methods were applied to the 1 μ L volume level in order to retain the sample. The additional methods included one 1 μ L set covered with 5 μ L of mineral oil overlay, made up of mostly alkanes to prevent evaporation, another set covered with 5 μ L of sealing solution overlay, a liquid of low viscosity also used to prevent evaporation, and the last set was covered with 5 μ L of sealing solution and then, after amplification, underwent MinElute (Qiagen) purification. MinElute is a spin column technique that uses a silica membrane for a bind-wash-elute process. DNA is absorbed into the membrane in high-salt condition (by buffer) as impurities are washed away. Low-salt buffer/water elutes the purified DNA off of the membrane. Part of testing the limitations of amplification volume is seeing any potential benefits when decreasing DNA input. The standard input that is used is 1 ng, but since the goal of this research is to eventually apply a developed optimized protocol towards trace input levels and possibly single cells, multiple input levels down to 5 pg, the DNA equivalent of a single cell, will be tested with different protocol parameters.

Additional Comparable Methods for Trace DNA

There are several other methods were tested and compared to reducing the amplification volume. One additional method is to compare more post-amplification purification strategies in combination with reduced volume. MinElute post-amplification purification has been described previously, but another kit to be used is the NucleoSpin gDNA Clean-Up XS (Macherey-Nagel), another silica membrane based post-amplification purification system. NucleoSpin follows the same idea as MinElute, in which DNA is bound to the silica membrane, the impurities are washed away, and the DNA is then eluted off of the membrane. The difference comes in to how low the elution volume can be to still allow quality profiles. The lowest optimal elution volume for MinElute is 10 μL . The lowest optimal elution volume for NucleoSpin is 6 μL . For this experiment, both purification methods will be carried out manually with MinElute at 5 μL and NucleoSpin tested at 5 μL and 3 μL elution volumes.

Another method that will be used in combination with reduced amplification volume will be testing different available thermal cyclers to determine if there is a more efficient way to amplify the samples. As a comparison to the GeneAmp thermal cycler, a Mastercycler ep Gradient S (Eppendorf) was available for use. The Mastercycler has the advantage of quicker ramp speeds over a standard thermocycler, shortening amplification program times by about half an hour to even an hour. Different amplification volumes and DNA inputs will be tested with the Mastercycler for any noted advantages.

Another one of the more popular methods used for trace analysis is to increase the PCR cycle number. Each of the previously mentioned DNA amplification kits, will have their PCR cycles increased from standard.

Single or Few Cells/Particles

Instead of being called cells, for this experiment, what is collected was referred to as particles. This is due to the fact that it is unknown what exactly is collected, as it could be dead cells or debris that may not have intact nucleases and therefore cannot be labeled as a cell (Figure 2).



Figure 2: Particles (circled in white) found on pair of shorts

Trace samples are collected via Gel-Film. This is a gel material placed on a polyester backing with low adhesion properties. This low adhesion allows for easy transfer of particles from clothing and other surfaces onto the gel. With no need to move the particles, the particles can be stained directly on the gel (Figure 3). A water soluble adhesive is collected onto the end of a tungsten needle (Figure 4), which is touched to the surface of the Gel-Film. The adhesive can easily lift the particle off, without disturbing other particles. This method is called micro-manipulation.

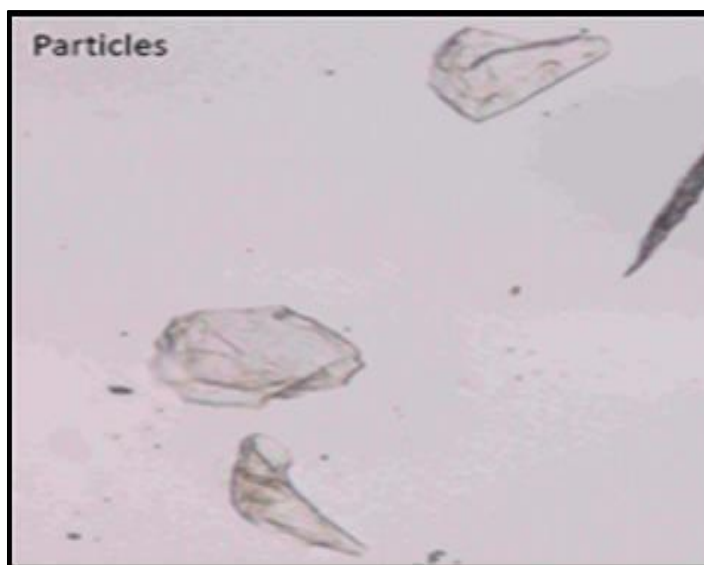


Figure 3: Particles stained directly on Gel-Film

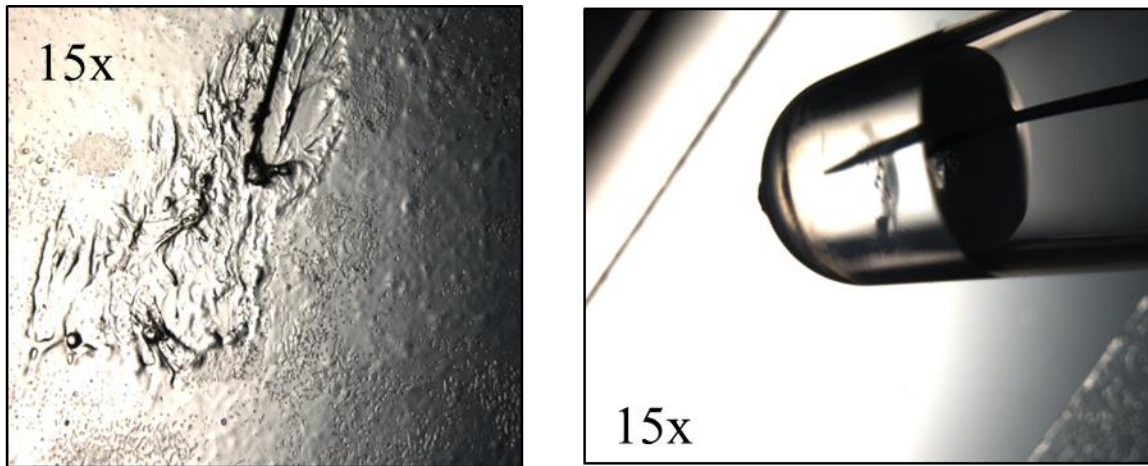


Figure 4: Collection of water soluble adhesive and, after particle collection, addition of adhesive into prepared amplification mix

Particularly for DNA collection, buccal slides will be used. Depending on which micro volume is the best suited for trace samples, that volume will be used along with comparison to standard volume. After collection from the slides, the cells/particles will be placed directly in amplification mix (Figure 4). This amplification mix uses the ForensicGEM lysis mix, which will break open any cells during amplification. The program to be used is 75°C 15 min (ForensicGEM activation), 95°C 11 min (Taq Gold activation), 34 cycles of 94°C 20 sec and 59°C 3 min, 60°C 10 min, and a 4°C hold.

Detection of STR Amplified Products

The method of STR detection was by capillary electrophoresis (CE). The instrument used was the 3130 Genetic Analyzer (Applied Biosystems). To prepare the plate, a master mix was made up of 9.7 μL of deionized formamide and 0.3 μL of size standard LIZ-500 per sample. Once mixed, 10 μL of this master mix was added to each well. About every 20-30 samples, 1 μL of a kit specific ladder was added as a labeling guide for numbering repeats within loci. 1 μL of sample was added to each well. A gray septa was placed on top of the plate and the plate was centrifuged for 30 seconds at 2000 rpm. A black plate base and white cover were attached to the plate and the plate was placed onto the 3130 instrument. After the electrophoresis was completed, the results were analyzed using GeneMapper software.

RNA Methodology

Sample Collection

A variety of body fluids were used to determine the effectiveness of RNA for tissue origin determination and the limits of the methodology. For blood samples, 50 μL was collected via venipuncture and was deposited onto sterile pieces of cloth. Semen samples were collected in 50 μL conical tubes. Swabs were placed into the conical tube and allowed to soak up sample. Saliva samples were collected by using a cotton-tip swab to rub the inside of the cheek. Vaginal and menstrual samples were collected by swabbing sides of semen-free vaginal cavity with a sterile swab. Only half of the semen and vaginal swabs were extracted. Skin samples were ordered pre-extracted.

Extraction

Organic extraction was used for these samples, carried out manually. A denaturing solution mix was created by 500 μL of denaturing solution and 3.6 μL of B-mercaptoethanol per sample. Of this mix, 505 μL was added to each sample and left in a 56°C water bath for 30 minutes. After centrifuged with spin basket, the samples and baskets were tossed out and 50 μL of sodium acetate and 600 μL of acid phenol:chloroform were added to each original sample tube. After centrifuging for 20 minutes, the upper phase was removed and placed in a new tube

while the bottom phase was discarded. 2 μ L of GlycoBlue carrier (Ambion), along with 500 μ L of isopropanol was added to each sample tube and placed at -20°C for an hour.

After the hour-long hold, the samples were centrifuged to form a blue resin at the bottom of the tube. This resin is the binding of the coprecipitant of the GlycoBlue carrier reagent to the RNA, with the blue dye serving as a visual aid for the pellet. The isopropanol was removed and replaced with 900 μ L of a 75% ethanol/ 25% DEPC-treated water wash. The samples were vortexed and centrifuged for 10 more minutes. The wash was removed completely and the samples were placed to dry in a vacuum centrifuge for 3-5 minutes. After drying, 20 μ L of nuclease free water was added to each sample and they were placed in a 60°C heating block for 10 minutes to allow for re-solubilizing of the pellet.

The samples were all treated with DNase via the Turbo DNA-free Kit (Ambion). 2 μ L of the TURBO DNase Buffer and 1 μ L of RNase-free TURBO DNase I were added to each sample. The samples were incubated in a 37°C water bath for 20-30 minutes. Afterwards, 2 μ L of the DNase Inactivation Reagent was added to each sample and vortexed. After a short centrifugation, a DNA pellet formed on the bottom of the tube. The supernatant was removed and placed in another tube while the DNA resin was discarded.

Quantitation

Quantitation was carried out via plate reader using the Quant-iT Ribogreen Assay kit (Invitrogen). Standards were made from a mix of 1X TE buffer and the RNA standard stock. The

standards ranged from 1000 ng/mL down to a blank sample. Samples were prepared by mixing 98 μ L of 1X TE buffer with 2 μ L of the RNA extract. The total volume, 100 μ L, of the standards and the samples were pipetted into wells on the plate. The RiboGreen mix, created by mixing 10 μ L of the Quant-iT Ribogreen reagent (Life Technologies) with 1.99 mL of 1X TE, was added directly on top of each sample, 100 μ L per sample. The plate was then placed into the Synergy 2 (BioTek) microplate instrument to quantitate the samples with a fluorescent emission setting of 535 nm and excitation setting of 485 nm. The concentrations of the samples were determined based on the standard curve created by the concentrations obtained for the standards.

Reverse Transcriptase Amplification (cDNA synthesis), RT-PCR

From the mRNA that was extracted, reverse transcriptase amplification is used to make complimentary DNA (cDNA). The High Capacity RT kit (Applied Biosystems) was used for this particular amplification. For the reactions, 14.2 μ L of sample and nuclease free water were combined. In order to get the amount of sample needed, the desired RNA input was divided by the sample concentration. This number was subtracted from 14.2 μ L to get the amount of water needed. The sample/water mixes were heated at 75°C for three minutes as the master mix was prepared. 5.8 μ L of master mix (10X RT buffer, 25X dNTP mix, 10X random primers, and Multiscribe RT) was added to each sample. For subsequent experiments, to determine limitations of reducing the volume, the RT volume was reduced to 10 μ L, 5 μ L, and 2.5 μ L. At first, the RNA input was held at standard 25 ng, but was also tested at 15 ng, 10 ng, 5 ng, and 1 ng. The

program used was 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes, and 4°C hold.

Body Fluid Multiplex Amplification

Through the body fluid multiplex amplification, fluorescently tagged body fluid specific primers are bound to the cDNA. The program used was 95°C for 15 minutes, 33 cycles of 94°C for 30 seconds, 55°C 90 seconds (+0.2°C per cycle), and 72°C for 45 seconds, then 72°C for 30 minutes ended by a 4°C hold. The standard volume overall is 25 µL (2 µL of cDNA sample and 23 µL of master mix (PCR mix, primer mix, Q-solution, and nuclease free water)) which all samples were tested initially along with standard volume and input with RT-PCR To determine limits of volume, the MP volume will also be tested at 12.5 µL, 5 µL, and 3 µL while RT-PCR volume is 20 µL and input is 25 ng. Other inputs at each volume will tested, which includes 10 ng and 5 ng. After these initial experiments, reduced RT-PCR volume and MP volume were tested together. The inputs tested were 25 ng, 10 ng, 5 ng, 1 ng, 0.5 ng, 0.25 ng, 0.1 ng, and 0.05 ng.

Touch Samples: Collection and Analysis

In order to obtain single and few cells for vaginal and saliva samples, cells were counted and diluted based on utilization of a hemacytometer. An extraction tube was filled with nuclease free water and a spin basket was placed inside. The swab was placed in the spin basket and swished vigorously. The tube was then centrifuged for 5 minutes and 14,000 rpm to get a pellet. The nuclease free water was removed from the tube and between 100 to 500 μL of fresh nuclease free water was added over the pellet. The tube was gently vortexed to disrupt and distribute the pellet throughout the water. A glass cover slip is placed onto the hemacytometer and 20 μL of the water/pellet mix is added to each side. Under a microscope, cells were counted within a 1 mm area of the grid. This was carried out for both sides and the two cell counts were averaged together. Since this count is in 10^4 cells/mL, adding a zero onto the average count would give cells/ μL (for example, the average cell count is 100, so the cells/ μL value would be 1000). Serial dilutions (1:2, 10 μL extract/10 μL nuclease free water) were made of the original water/pellet mix down to 1 cell/ μL . Each of the dilutions went through the standard extraction and quantification methods. For RT-PCR and the MP amp, a comparison was made between standard volumes and reducing the volumes of both.

Touch swabs were collected for skin samples. The swabs were lightly dampened with nuclease free water, and vigorously swabbed over areas of potential “touch”. During extraction, several conditions were used for comparison. One set was carried out without the DNase treatment. The second set was carried out with the DNase step but with an elution volume of 10 μL . The last set was carried out following the standard extraction. As what was done with DNA

samples, it was of interest to determine if, in combination with reduced volume, increasing the amplification cycle numbers during the MP amp would improve detection of body fluid markers. When carrying out RT-PCR, two groups of the samples were made, one group with standard volume and the other with reduced volume. For the MP amp, these two groups were split into two more groups each, with one of the new groups at standard cycle number, and the other group was increased to 35 cycles.

An additional method was carried out on these touch skin samples. Pre-amplification has been suggested as a step to use before the MP amp in order to increase the amount of template of cDNA for binding of body fluid gene primers. The pre-amp step used only one primer, LCE1C for skin, in order to amplify the amount of the LCE1C template in the sample, if there is any at all. 3 μ L of the RT-PCR product is used, with 22 μ L of master mix for a total volume of 25 μ L. The program for the pre-amp step was 95°C for 5 minutes and 10 cycles of 95°C for 15 seconds and 60°C for 2 minutes. For this program, like the Identifiler Plus program, the anneal and extend process is combined into one step. Again, to compare all possibilities, a second set of pre-amp samples were purified using MinElute (as previously described in DNA methodology).

Cell/Particle Collection and Analysis

In the same manner as DNA collection cell collection, skin particles will be collected from five sources in groups of 10, 25, 50, 100, and 0 (as a blank). Reduced volume RT (5 μ L total) master mix was prepared with 3.55 μ L of nuclease free water and without Multiscribe

enzyme. As particles are collected, they will be placed directly into the RT master mix, heated, and the enzyme was added before amplification. Unlike before, 5 uL of sample will be used for the pre-amp, with 14 cycles, and 5 uL of pre-amp product will be used in a standard MP volume amp. This increase is to accommodate for the minuscule amount of intact cells among the collected particles. This same experiment will be carried out for clump collection as well. Clumps are particles bunched together in a single grouping. The clumps will be collected in groups of 1, 5, 10, 20, and 0 (as a blank).

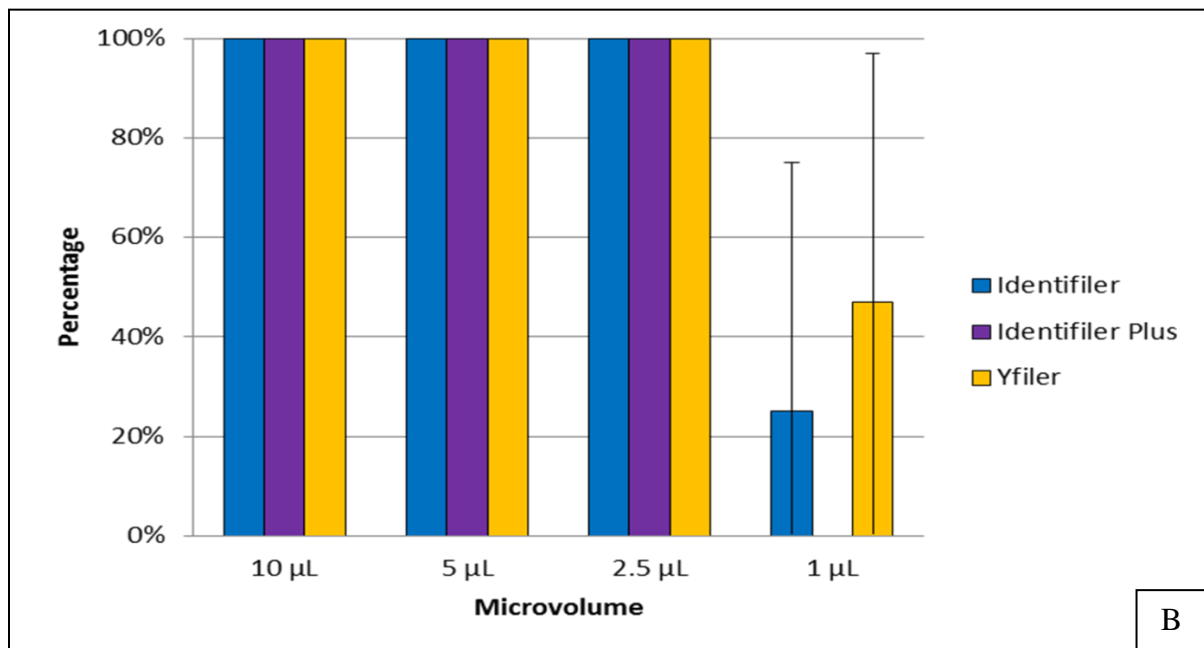
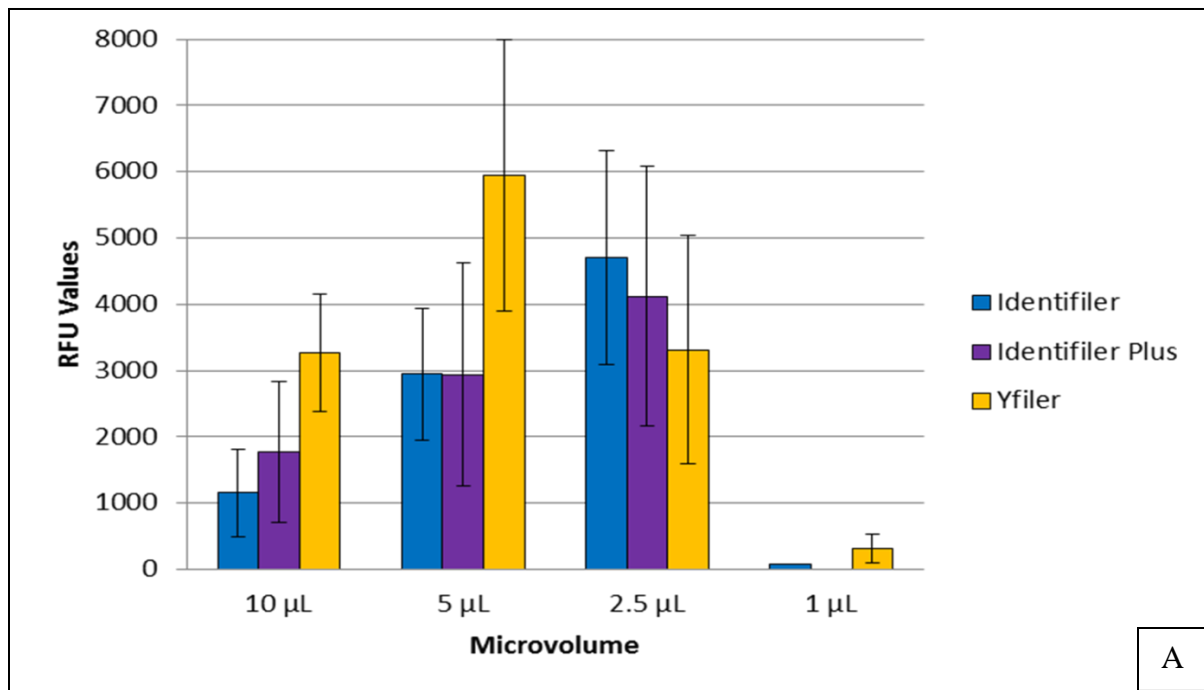
Detection of Body Fluids

Detection of body fluids followed the same procedure as detection and analysis of STR amplified products, except without use of a ladder.

CHAPTER THREE: RESULTS

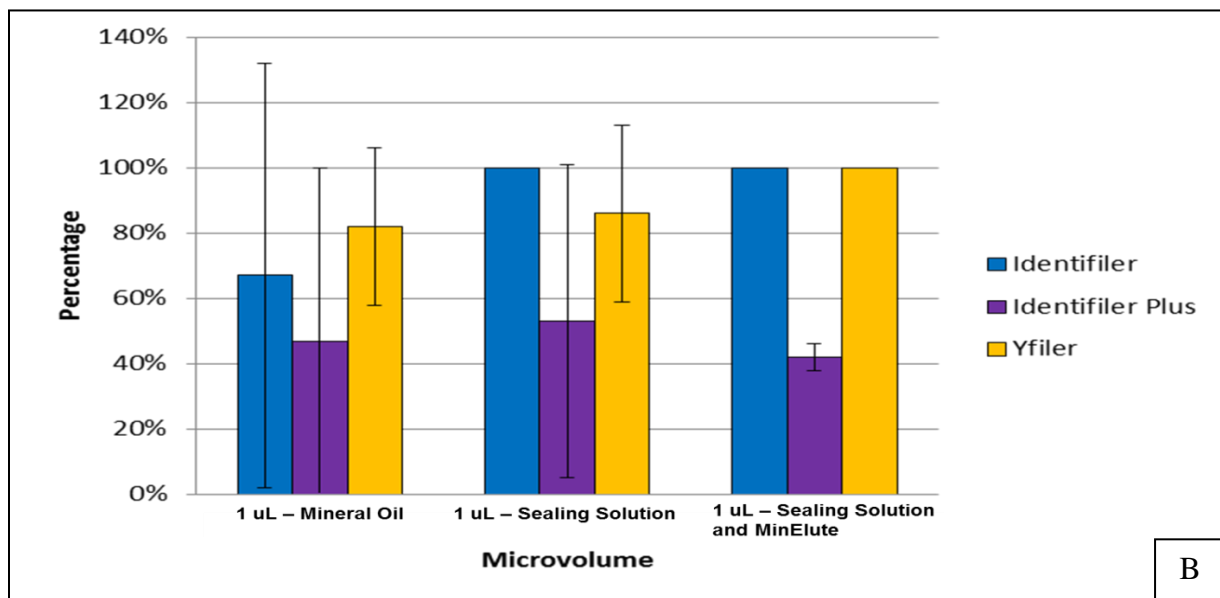
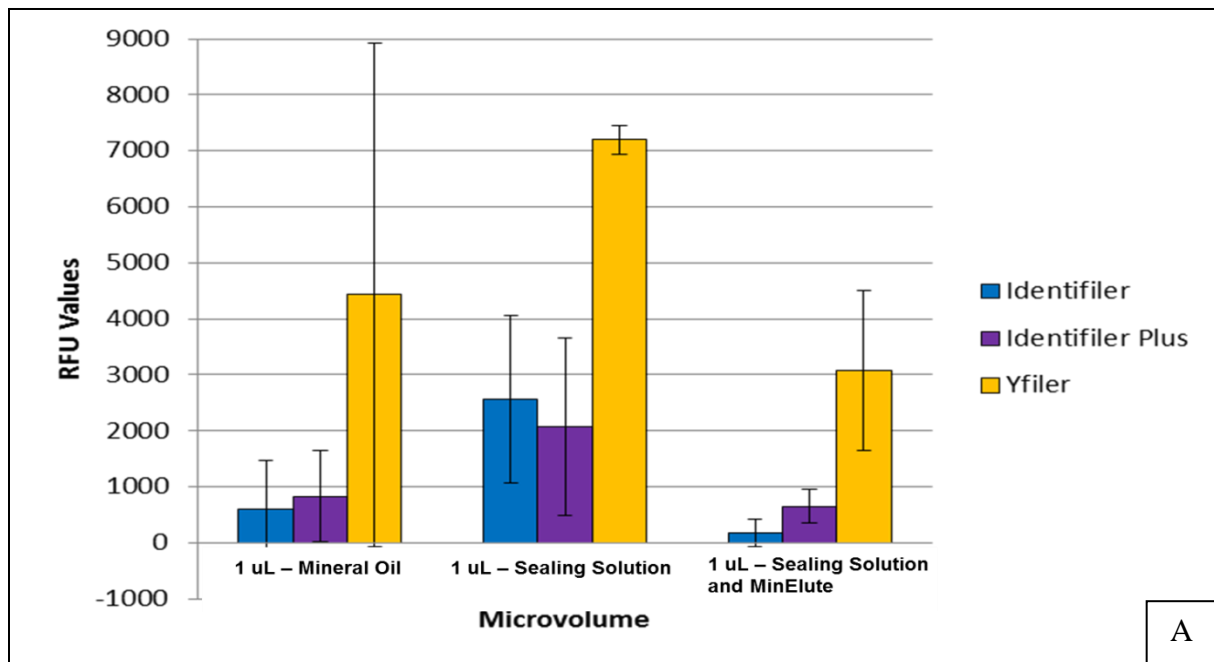
DNA Results

For the initial experiments, DNA input was held at 1 ng as the PCR volume was reduced from 25 μ L to 10 μ L, 5 μ L, 2.5 μ L, and 1 μ L. The samples that were used three male 50 μ L bloodstains. Figure 5 shows the profile recovery percentage for each kit used at each PCR volume. Between 10 μ L and 2.5 μ L, full profiles for each of the samples used were recovered. The issue comes up when approaching the 1 μ L volume, which was expected when taking into consideration evaporation. To accommodate for this, three other methods for the 1 μ L volume were examined and compared. One set used a 5 μ L mineral oil overlay, the second set used a 5 μ L sealing solution overlay, and the last said used a 5 μ L sealing solution overlay in combination with post-amplification purification via MinElute. By adding an overlay, profile recovery immediate increased for all kits (Figure 6). For experiments involving reducing the DNA input amount, it was decided that 1 μ L with sealing solution only would be the best choice as it provided good results and the highest relative fluorescent units (RFU's).



(N = 3) Comparison of (A) RFU values and (B) profile recovery for reduced PCR volumes.
 Note: no profile was detected for any of the three male 50 µL bloodstain with the Identifiler Plus kit

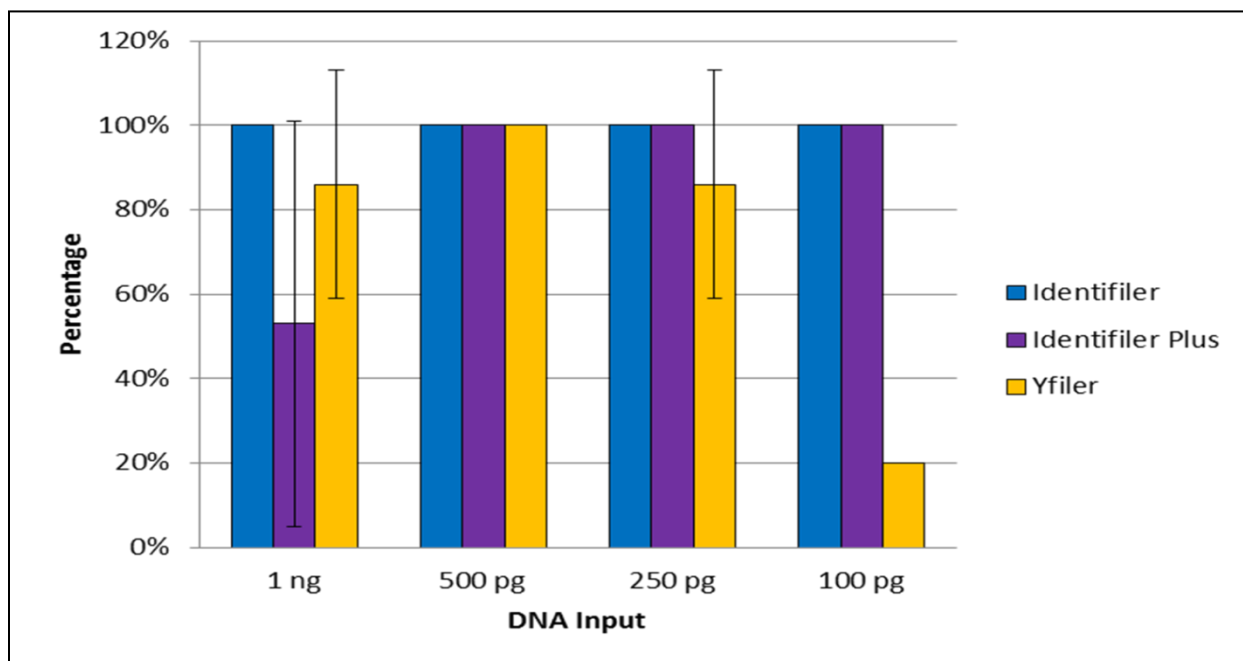
Figure 5: Comparison of RFU values and profile recovery for reduced PCR volumes



(N = 3) Comparison of (A) RFU values and (B) profile recovery for three additional methods for 1 μ L PCR volume. The samples used were the same three previously used male 50 μ L bloodstains.

Figure 6: Comparison of RFU values and profile recovery of three 1 μ L PCR volume methods

After establishment of using the 1 μL volume with a 5 μL sealing solution overlay, it was essential to determine the limits of detection with the same three male 50 μL bloodstain samples. Aside from the standard 1 ng DNA input, 0.5 ng, 0.25 ng, and 0.1 ng were also tested. The RFU values were high, around the same values as dictated in Figure 6, and profile recovery for the lower inputs were high, except for Yfiler, which dove down to about 20% recovery with 0.1 ng DNA input (Figure 7). This same experiment was tested with saliva and semen, to make sure that there were no discrepancies between body fluids. The results followed the aforementioned experiment results, except that Yfiler followed the other two kits in profile recovery.

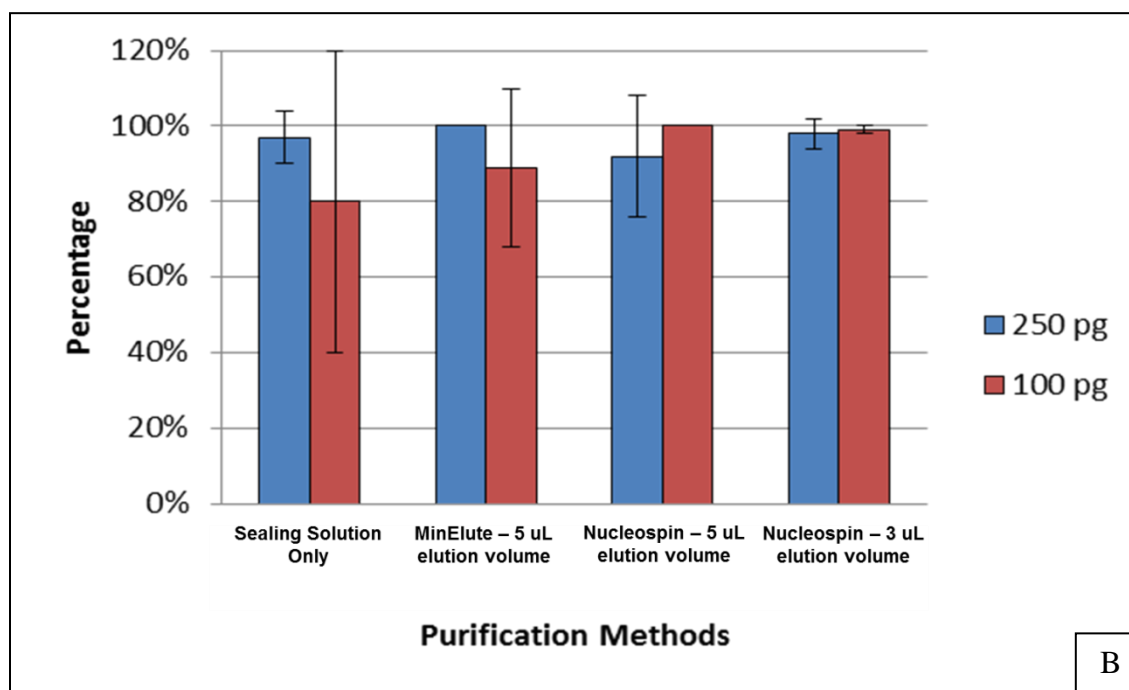
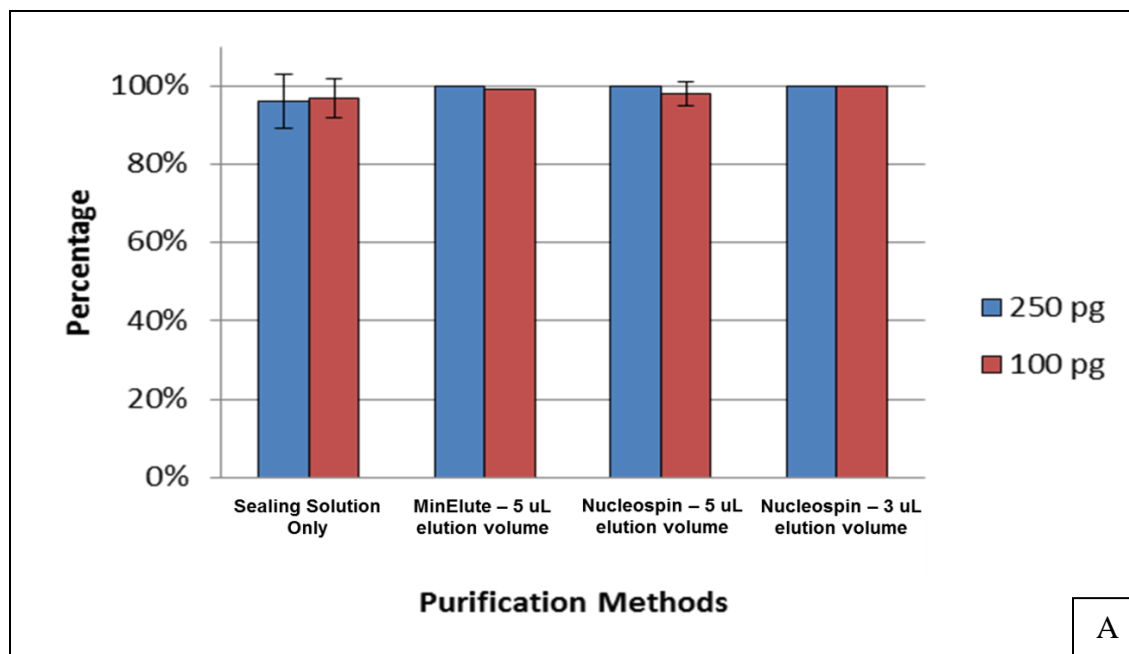


(N = 3) Comparison of DNA inputs and profile recovery with 1 μL amp volume with a 5 μL sealing solution overlay.

Figure 7: Comparison of profile recovery of reduced DNA inputs at 1 μL PCR volume

An issue arose with using 5 μL of sealing solution only. This issue comes when removing 1 μL of sample for the capillary electrophoresis preparation. The reaction mixture, forming a “bubble-like” appearance, for the amp is hardly visible when sealing solution is overlaid. This causes issues when wanting to only take out the 1 μL reaction mixture and not any of the sealing solution, which would cause problems with profile detection. Even when it’s possible to successfully remove 1 μL of reaction mixture only, it still requires extra time to find exactly where the reaction mixture “bubble” is, which is not ideal in a fast paced forensic laboratory. It was decided upon at this point to reexamine the use of post-amplification purification methods.

Ten samples, including four 50 μL bloodstains, three saliva swabs, and three semen swabs, with 1 μL reaction volumes with 5 μL sealing solution overlay combined with MinElute purification with an elution volume of 5 μL was tested at 0.25 ng and 0.1 ng. NucleoSpin post-amplification purification was also tested for comparison at 0.25 ng and 0.1 ng, with two elution volumes of 5 μL and 3 μL . Unlike with previous experiments, only Identifiler Plus and Yfiler were tested. The reason for choosing Identifiler Plus was that the results were reliable, with good RFU values and profile recovery, but also that the kit is beginning to be picked up more frequently within forensic laboratories over the usual Identifiler kit due to several more ideal factors, including shorter amplifying time and it being designed to decrease artifacts, and improved profile quality and recovery with degraded and low amount samples.



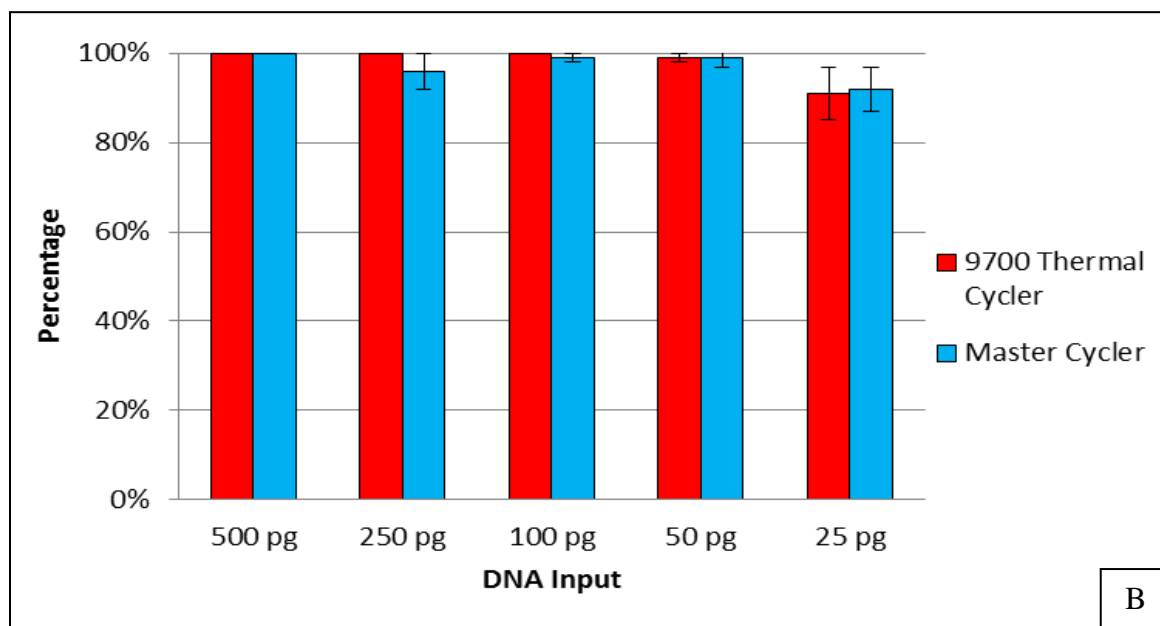
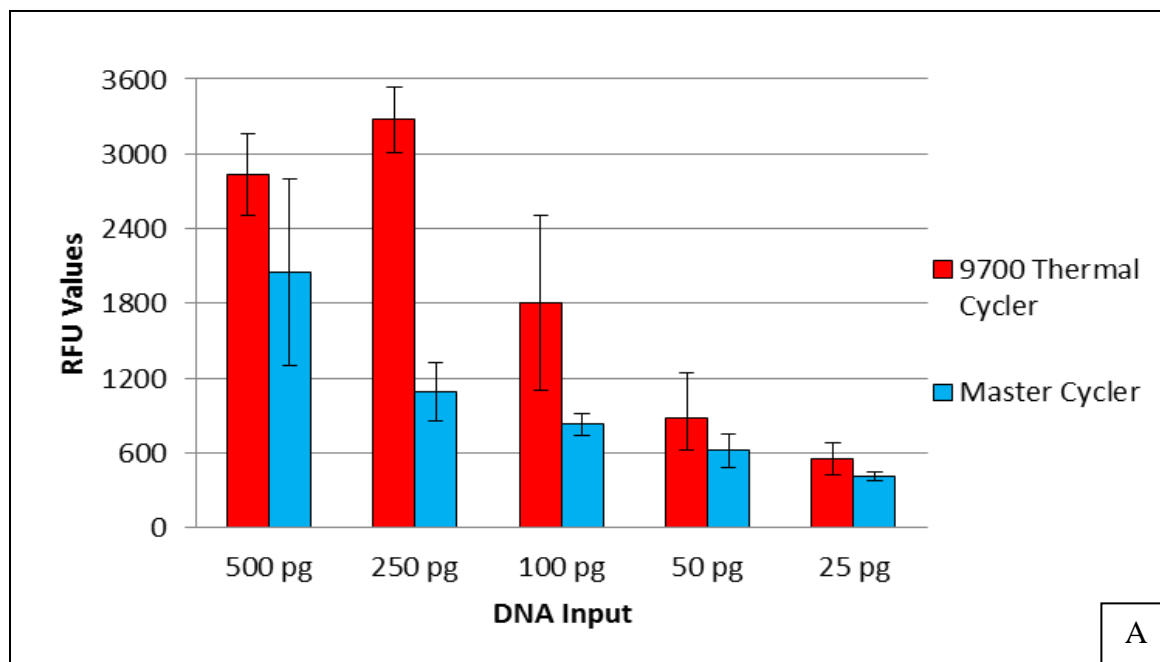
(N = 10) (A) Identifiler Plus, (B) Yfiler. Comparison of profile recovery percentage between multiple post-amp purification methods.

Figure 8: Comparison of profile recovery of multiple post-amp purification methods at 1 μ L PCR volume

The RFU values for all of the samples were generally higher for the samples with sealing solution only, but for Identifiler Plus, NucleoSpin with a 3 μ L elution volume had similar RFU values and had one of the highest percentages of profile recovery of the four methods. For Yfiler, the RFU values dipped below 1000 for MinElute, and gradually increased to NucleoSpin with the 3 μ L elution volume. Although it was still lower on average than sealing solution only, the values were still acceptable with the highest profile recovery (Figure 8). At this point it was decided to move ahead with NucleoSpin purification at a 3 μ L elution volume.

These same samples were tested again with NucleoSpin purification and 3 μ L elution volume. The DNA inputs tested were 0.5 ng, 0.25 ng, 0.1 ng, 0.05 ng, and 0.025 ng. There was access to an Eppendorf Mastercycler at this point in time. Due to the advantages that were said to come with the Mastercycler, these DNA inputs, with both Identifiler Plus and Yfiler kits, with NucleoSpin purification were also tested on the Mastercycler for comparison to the standard thermal cycler.

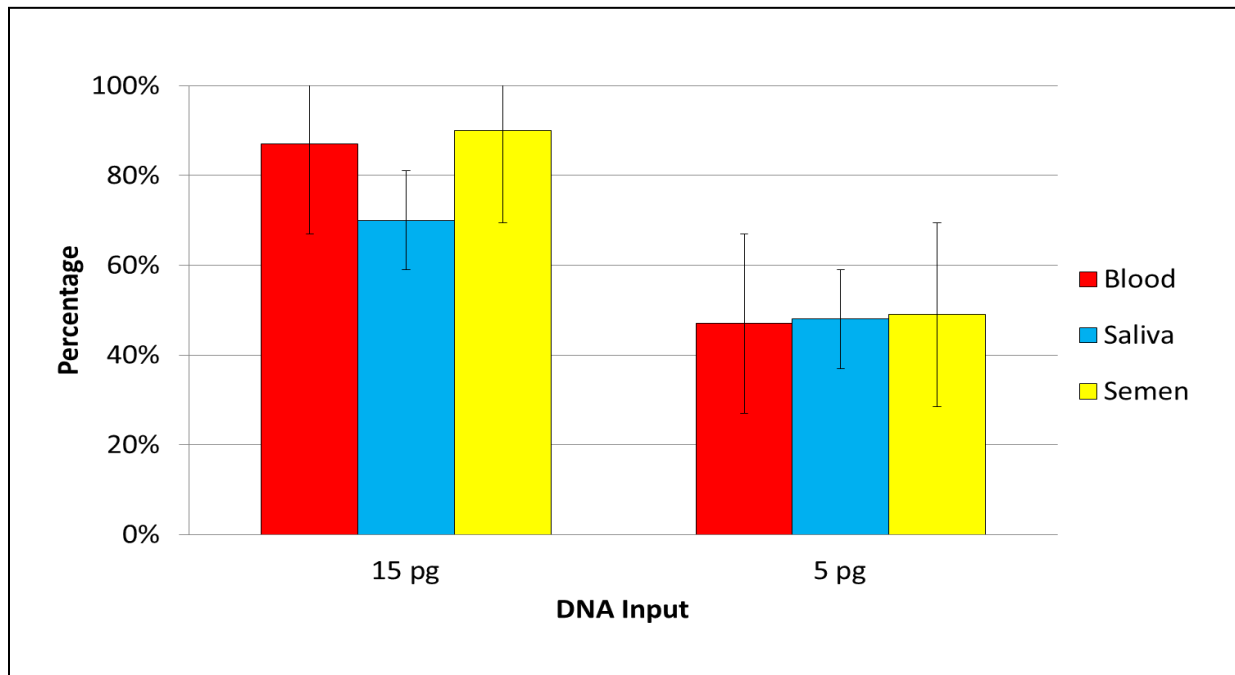
The average RFU values for both Identifiler Plus and Yfiler were much lower with the Mastercycler. Although there wasn't much of a difference in profile recovery, less full profiles were obtained when using the Mastercycler on average for both kits when compared to the standard thermal cycler (Figure 9). Even though the Mastercycler has the advantage of faster ramping speeds, which in turn lead to shorter program times, it's not enough of an advantage to choose over standard instrumentation, so the 9700 thermal cycler was chosen for subsequent experiments.



(N=10) Identifiler Plus is shown only. (A) Average RFU Values, (B) Percentage of profile recovery

Figure 9: Comparison of RFU values and profile recovery for two separate thermal cyclers (Identifiler Plus shown only)

The input was decrease further to 0.015 ng and 0.005 ng (the equivalent to a single cell). These inputs were tested on the standard thermal cycler, using Identifiler Plus and Yfiler kits, with a 1 μ L reaction volume, overlaid with 5 μ L of sealing solution, and post-amp purified with NucleoSpin and an elution volume of 3 μ L. The profile recovery at these inputs, while using reduced volume, is around 70-80% for 0.015 ng and around 40-60% for 0.005 ng (Figure 10). The Identifiler Plus profile of a blood sample is shown in Figure 11. The profile is at the input of 0.005 ng, the DNA equivalent of a single cell.



(N=10) Profile recovery for Identifiler Plus. Similar results were obtained for Yfiler

Figure 10: Profile recovery at the DNA equivalent to single and few cells

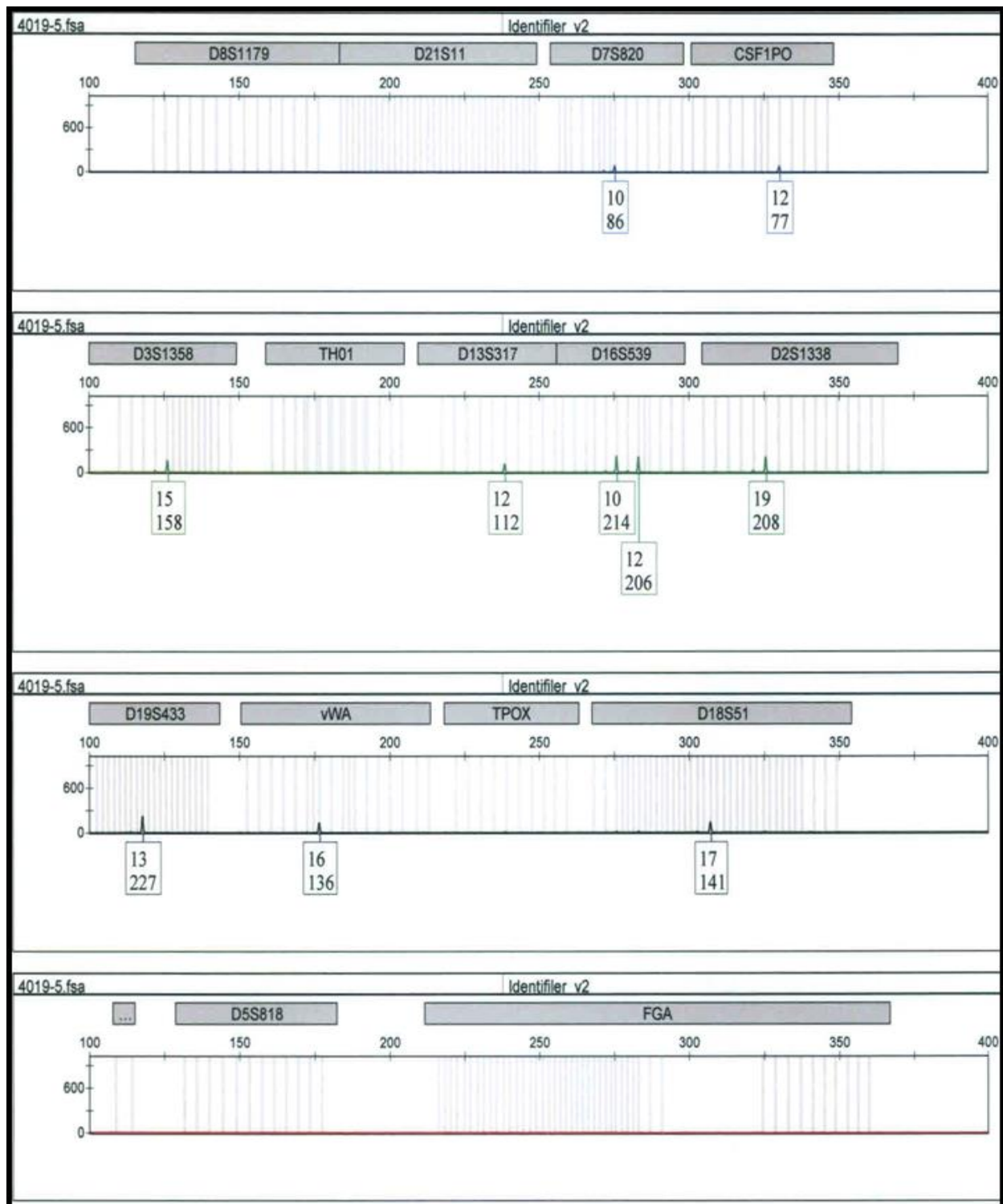
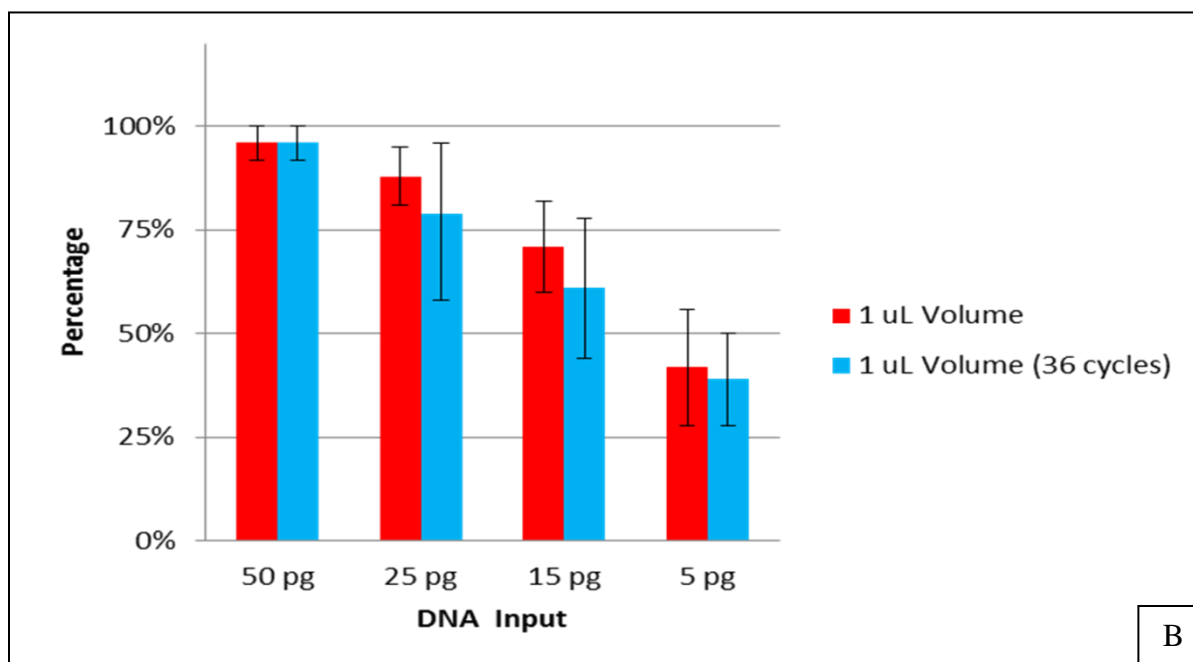
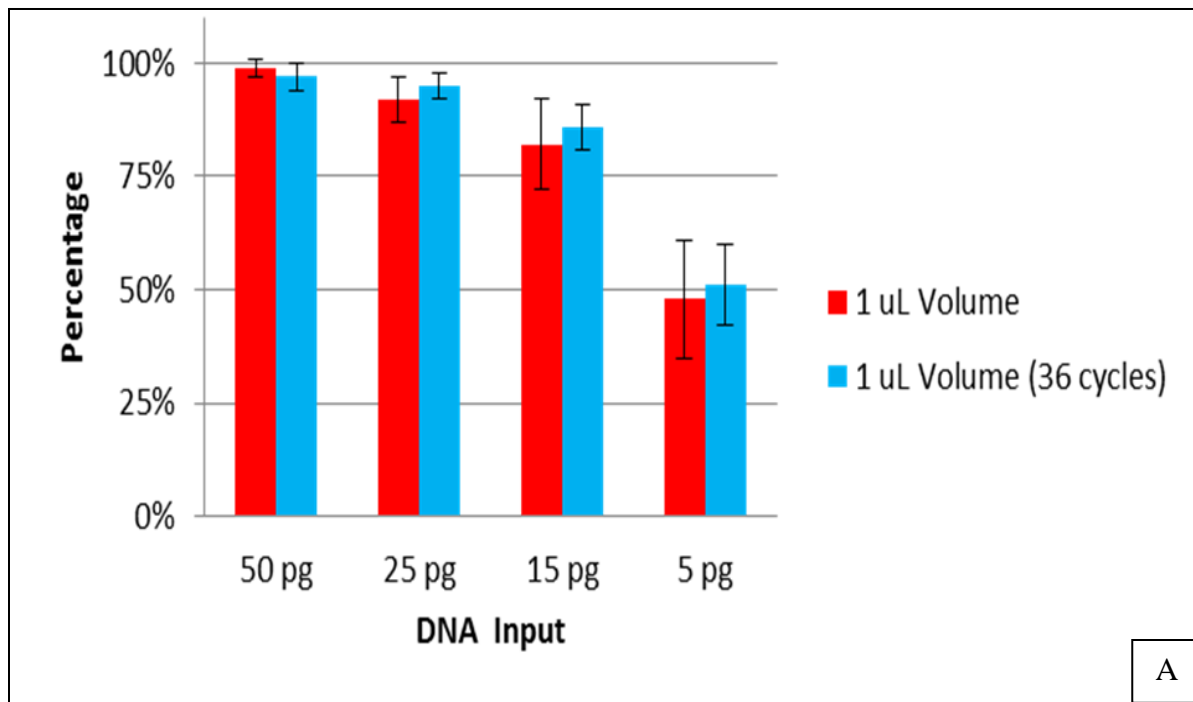


Figure 11: Identifiler Plus profile of blood sample at 0.005 ng, the DNA equivalent of a single cell

As alleles were recovered at 0.005 ng, it was important at this point to try to increase the recovery percentage (try to recover more alleles and increase RFU values). As mentioned before, a common practice to increase profile quality is to increase the PCR cycles during amplification. For both kits, cycle numbers were increased to 36. The reaction volume was at 1 μ L with a 5 μ L sealing solution overlay, and NucleoSpin purification with a 3 μ L elution volume. The inputs tested were 0.05 ng, 0.025 ng, 0.015 ng, and 0.005 ng.

Profile recoveries for Identifiler Plus samples were similar between the standard and increased cycle number. With increased cycles, average recovery was slightly higher, but not enough to really show a major advantage. Recovery for Yfiler was similar as well, on average, between standard and increased cycles, with standard being slightly higher with each input. As expected, the average RFU values were much higher with increased cycle number, but that also came with an increase in pull-up, allelic drop-in, and noise (Figures 12, 13).

Since increasing PCR cycles did not show much improvement, it was necessary to see what other improvement can be made to this process. One way to improve the process is to cut out unneeded steps. Instead of continuously purifying after amplification, it may be possible to achieve the same results at a slightly increased reaction volume, which would save time and extra materials such as sealing solution, without using too much sample or master mix reagents. One reduced volume set that tended to do well without the need of sealants was the 2.5 μ L.



(N=10) Comparison of standard and increased cycle number profile recovery of (A) Identifiler Plus, (B) Yfiler

Figure 12: Comparison of profile recovery at standard and increased PCR cycle number

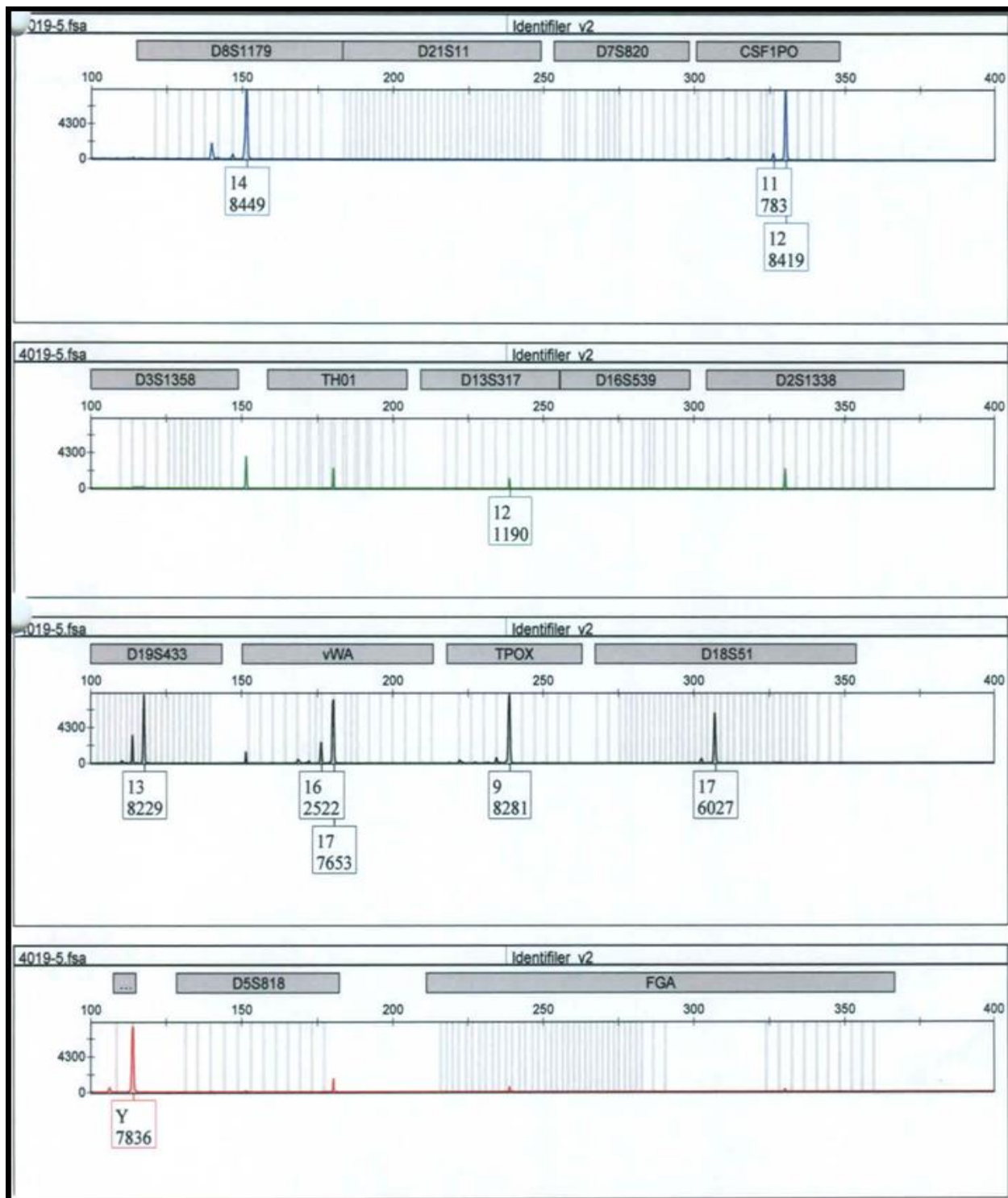
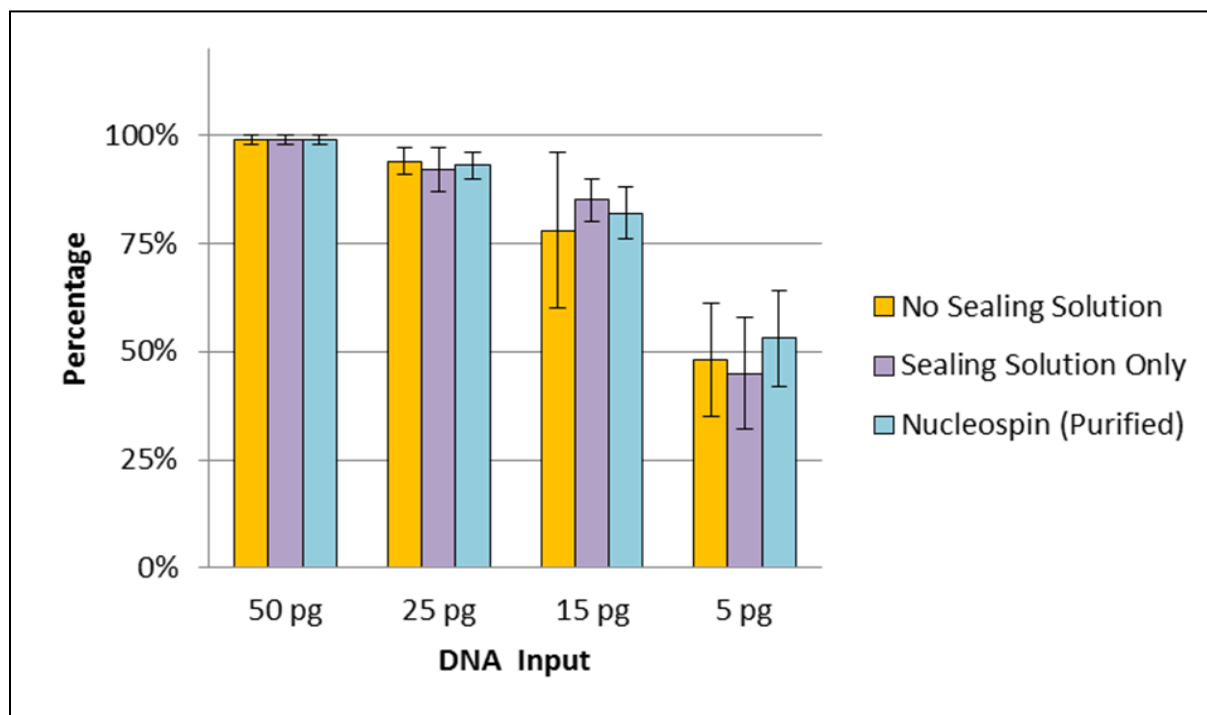


Figure 13: Identifiler Plus profile of blood sample at 0.005 ng, the DNA equivalent of a single cell, with increased PCR cycles (36)

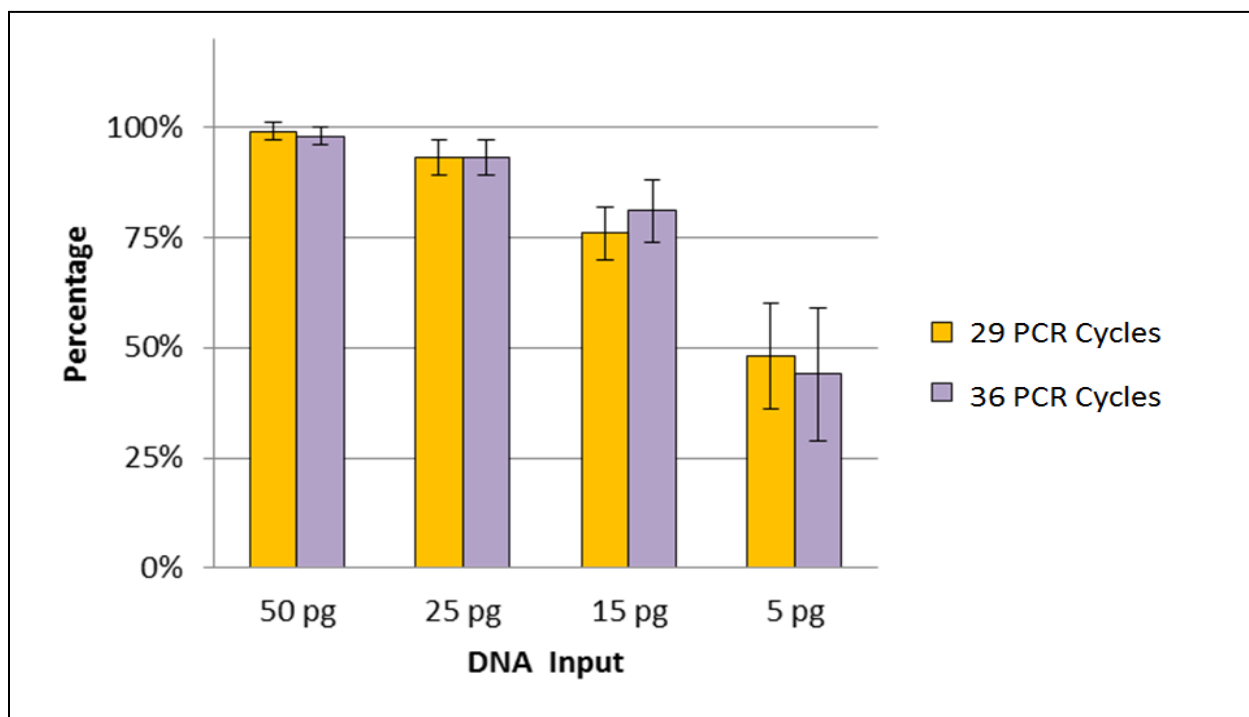
The reaction volume was set at 2.5 μL , only tested with Identifiler Plus due to time. Three sets were tested to determine the feasibility of using only 2.5 μL . The first was only the sample and master mix without any overlays or purification. The second set had a 4 μL sealing solution overlay but no purification. The last set had a 4 μL sealing solution overlay with NucleoSpin purification at a 3 μL elution volume. The inputs used were 0.05 ng, 0.025 ng, 0.015 ng, and 0.005 ng. Figure 14 shows the profile recovery for each set. The results with no sealing solution are very similar to the other two sets, with nearly 100% recovery at 0.05 ng and 0.025 ng, and about 50% recovery at 0.005 ng.



(N=10) Comparison of the profile recovery of three amplification methods at a reaction volume of 2.5 μL

Figure 14: Comparison of profile recovery with multiple 2.5 μL PCR volume methods

The 2.5 μL reaction volume was also tested with increased PCR cycle numbers (36). This was just to confirm whether or not increasing the cycles would have an effect on any volume that is chosen, and wasn't just isolated to the 1 μL volume sets. Like the previous experiment, 0.05 ng, 0.025 ng, 0.015 ng, and 0.005 ng were tested at the 2.5 μL volume. No sealing solution or purification were carried out, only the increase in amplification cycles.

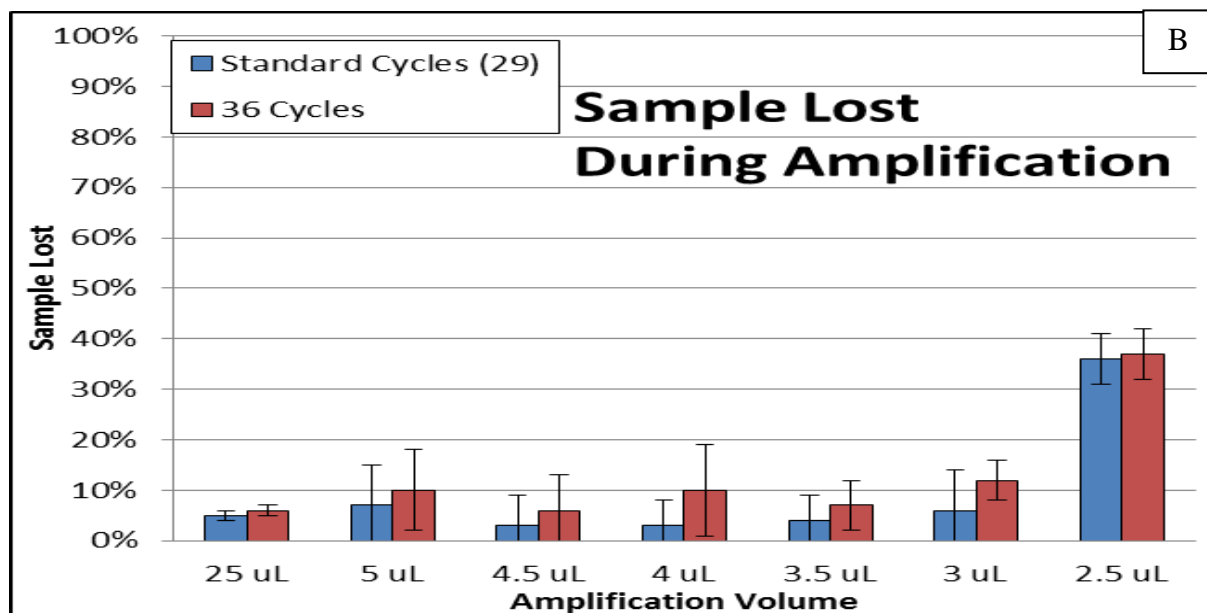
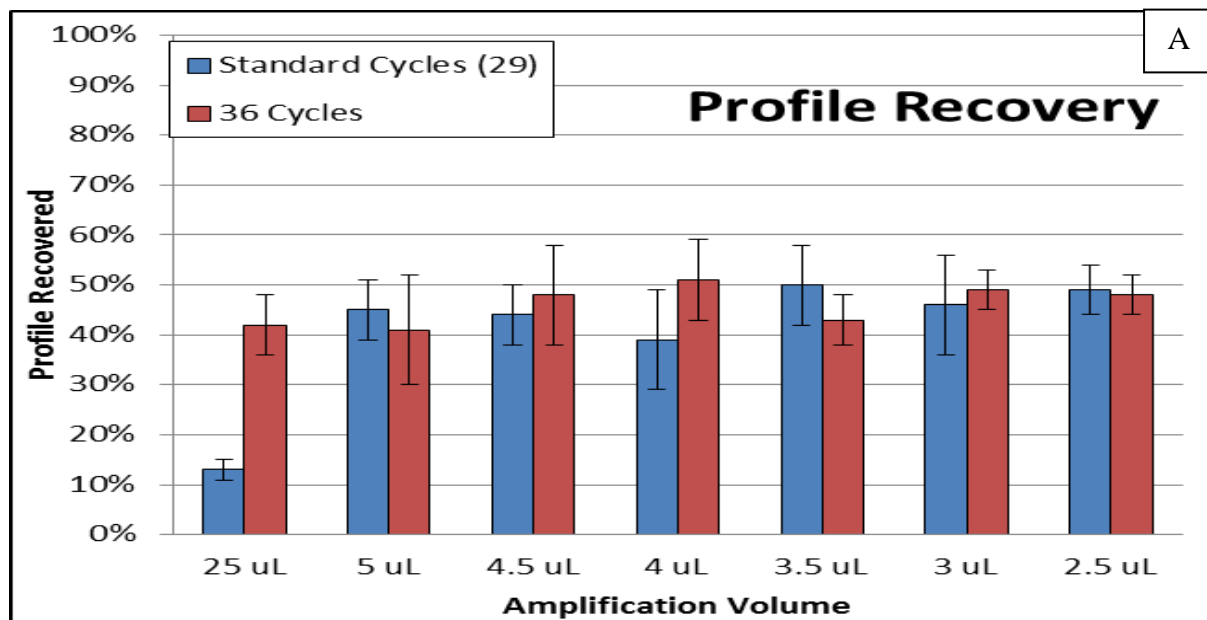


(N=10) Comparison of the profile recovery between standard and increased amplification cycle numbers at 2.5 μL reaction volume

Figure 15: Comparison of profile recovery with standard and increased amplification cycles at 2.5 μL PCR volume

As shown by Figure 15, the profile recovery between standard 29 cycles and increased 36 cycles is very similar, with standard being slightly higher on average (exception at 0.015 ng). Due to using such low volumes, there was concern over evaporation rates since sealing solution wasn't used anymore. This may be preventing even higher profile recovery at few cells or single cell DNA input level. In order to determine the best micro volume with minimal evaporation and with similar results to the 2.5 μ L reaction volume, multiple volumes were tested, starting with standard down to 2.5 μ L. The input level tested was 0.005 ng to get the best idea on how single cells may be affected by evaporation levels and which micro volume would be the most ideal overall.

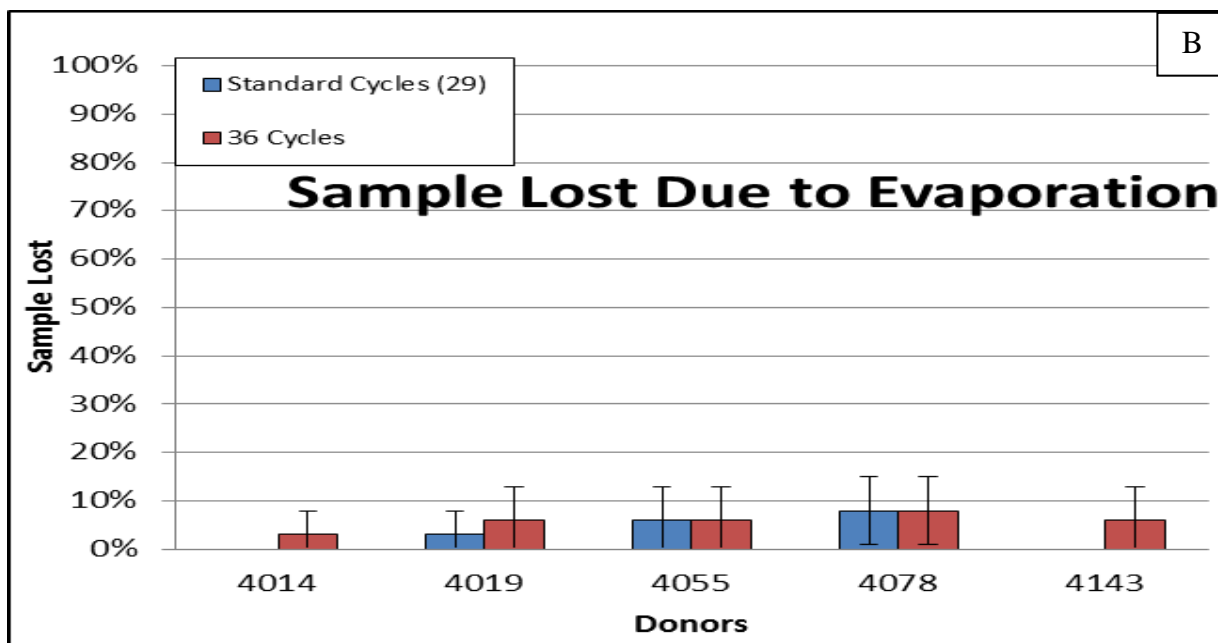
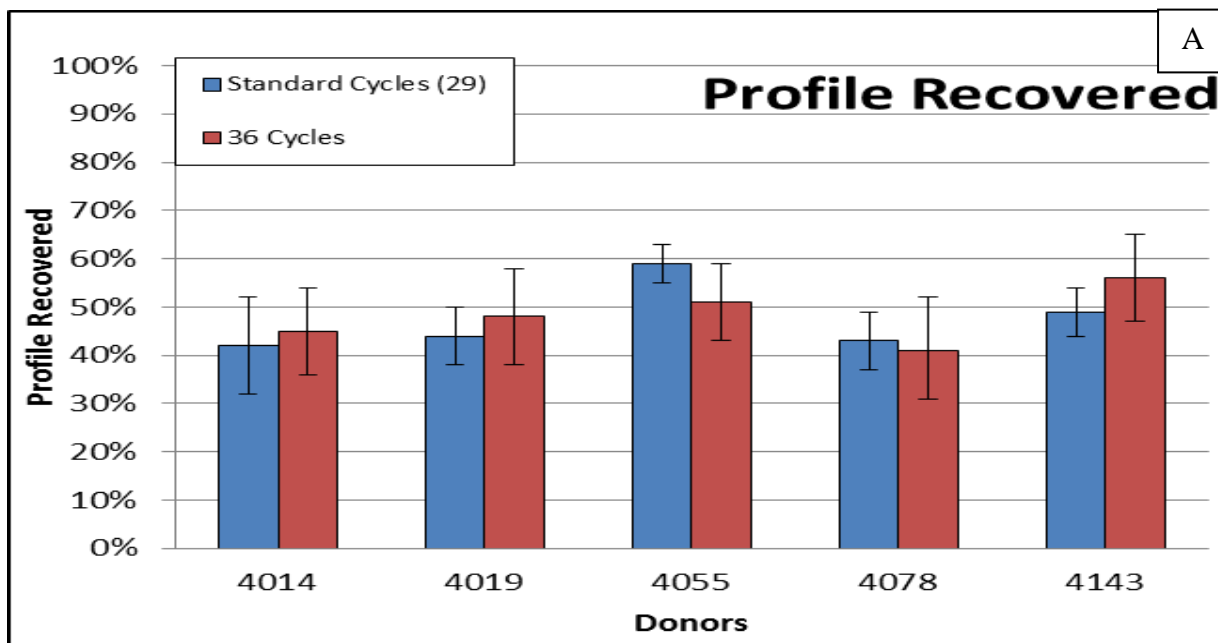
When looking at the 2.5 μ L volume, in Figure 16, evaporation is very high, between 30-40% of sample is lost during amplification. Due to such a high evaporation rate, which can affect the results, the 2.5 μ L is not the most ideal micro volume to work with. When comparing all of the micro volumes tested in this experiment, one volume in particular stands out due to its results when following the standard number of amplification cycles. The 3.5 μ L reaction volume, when following the standard cycles, has one of the highest rates of profile recovery when comparing to both standard and increased cycles, and had the highest rate of profile recovery among standard cycles. It is also important that evaporation rates are low for this to be a successful volume, and the 3.5 μ L volume has one of the lowest evaporation rates, around 3% with standard cycles, and about 7% with increased cycles of overall sample loss.



(N=20 for 25 μ L and 2.5 μ L, 5 for 3 μ L-5 μ L) Comparison of multiple volumes at standard cycles and increased cycles for (A) profile recovery and (B) evaporation rates

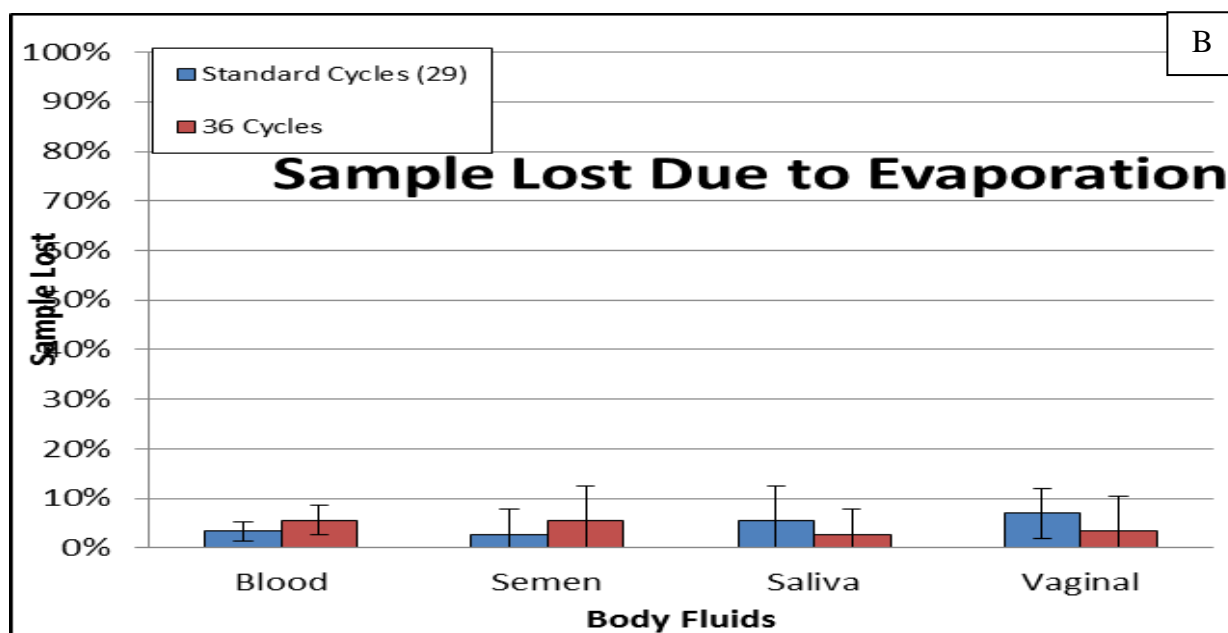
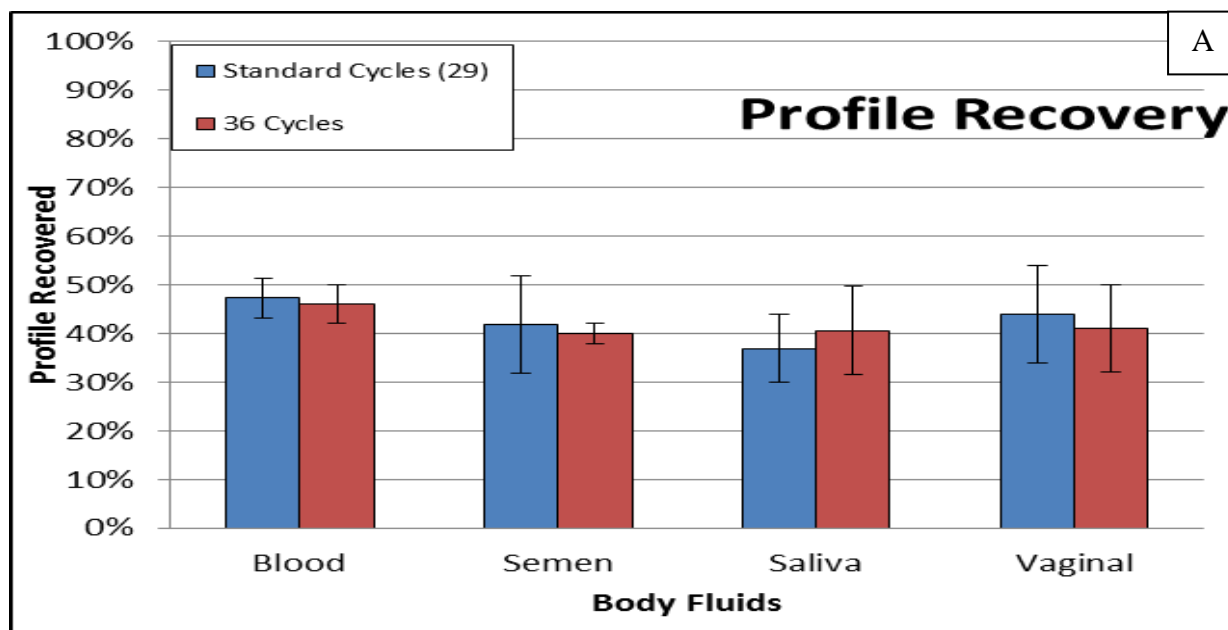
Figure 16: Comparison of profile recovery and evaporation rates for multiple micro volumes at standard and increased PCR cycles

To determine the validity of the results, five more blood samples were tested at 3.5 μL reaction volume and an input of 0.005 ng. Multiple body fluids were also tested to determine if there was any discrepancy between the tissue origins. For the extra blood samples (Figure 17), on average, profile recovery was between 40% and 60%, which lines up with the previous results. Evaporation rates also followed the previous results as sample lost ranged between 0% and about 8% for both standard and increased cycle numbers. When looking at Figure 18 in comparison of body fluids, most of the profile recovery averages were over 40%, with one average (saliva) dipping slightly below 40% (~37%). The evaporation rates for each body fluid, though, remained around the same values as before, with about 2% to 8% of sample lost on average. Results for using 3.5 μL as a viable micro volume show promise for the future of adaptation to the few/single cell level.



(N=5) Additional testing of 3.5 μ L reaction volume with five extra blood donors. (A) Profile recovery and (B) Evaporation rates

Figure 17: Comparison of profile recovery and evaporation for additional blood donors at 3.5 μ L

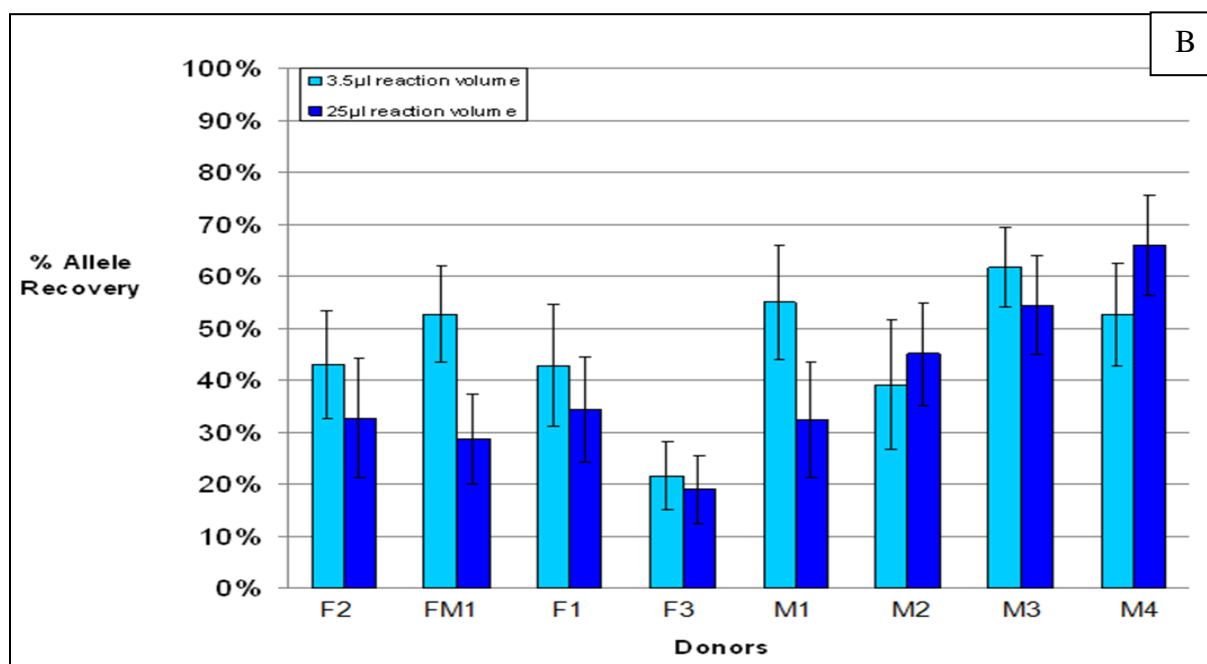
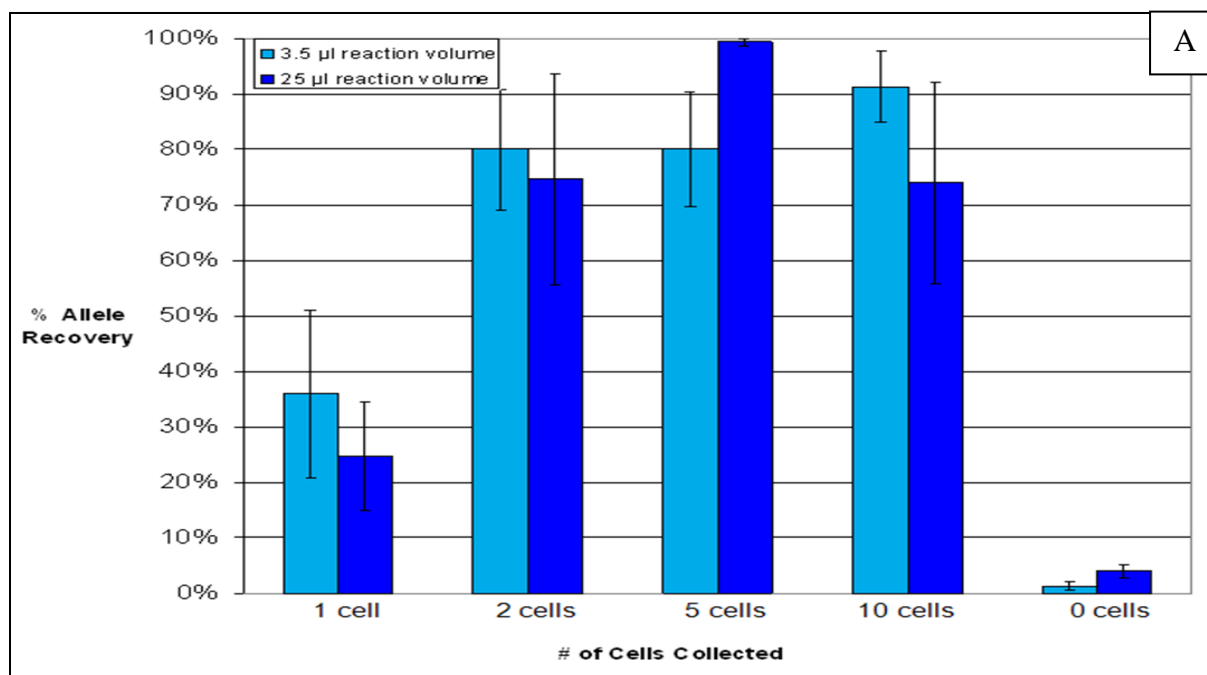


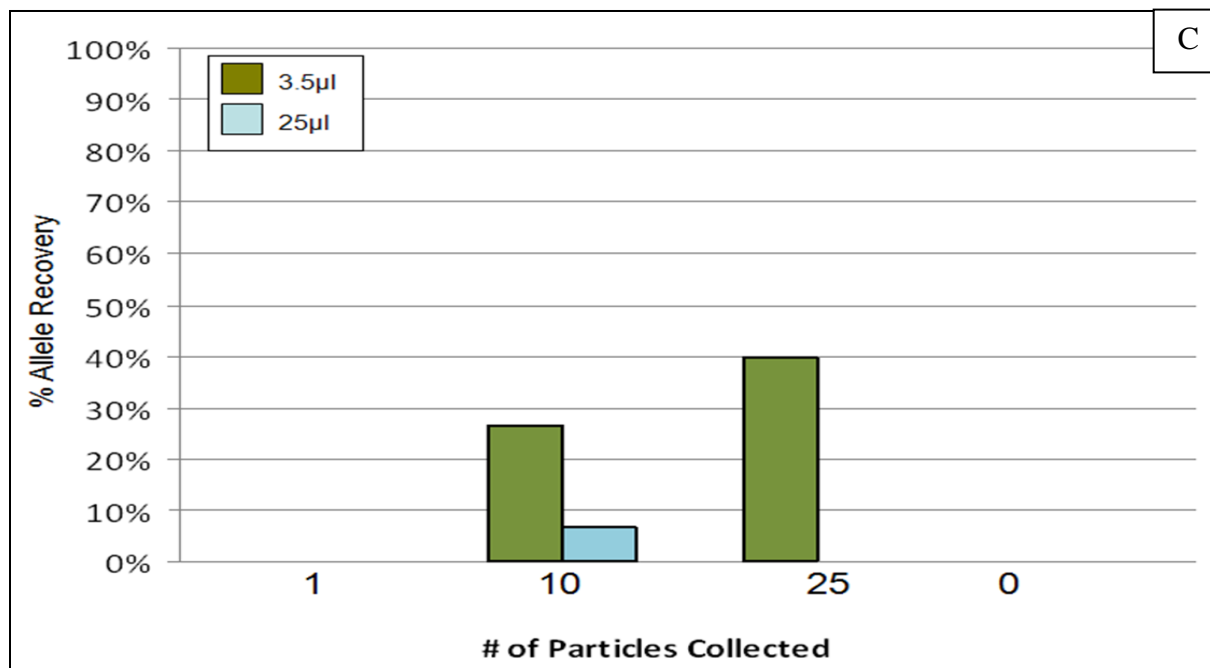
(N=5 for blood, 1 for semen, saliva, and vaginal with five replicates) Body fluid comparison at 3.5 μ L. (A) Profile recovery and (B) Evaporation rates

Figure 18: Comparison of profile recovery and evaporation for multiple body fluids at 3.5 μ L

As described in the DNA methodology section, buccal slides were collected and through micro-manipulation, cells/particles were removed from the slides. Since 3.5 μL was chosen as the most ideal volume to work with, a master mix was developed with 1.4 μL of PCR reagent, 0.7 μL primer mix, and 1.4 μL of ForensicGEM lysis mix (made by 6.9 μL sterile water, 2.1 μL 10X buffer, and 1.0 μL ForensicGEM enzyme). Collected samples were also tested at standard volume, using 10 μL of the lysis mix.

Looking at Figure 19A, for most of the cell counts, reduced volume showed higher profile recovery over standard volume. At one cell, there was a 40% profile recovery rate as opposed to about 25% obtained with standard volume. There was even an 80% profile recovery with only two cells. With single cells collected from multiple donors, in Figure 19B, on average, there was an increase in profile recovery with reduced volume of about 10-20% over standard volume. Particles, as opposed to buccal cells, were also collected from a coffee cup grip. With 25 particles, 40% of a profile was recovered at standard volume, with no profile obtained at standard volume (Figure 19C). Figure 20 shows the profile obtained at 25 particles from the coffee cup grip.





(N=5 for (A), 8 for (B), 1 for (C)) Profile recovery of (A) collected buccal cells, (B) single buccal cells collected from multiple donors, and (C) particles collected from a coffee cup grip

Figure 19: Comparison of profile recovery with single cell and few cell collections

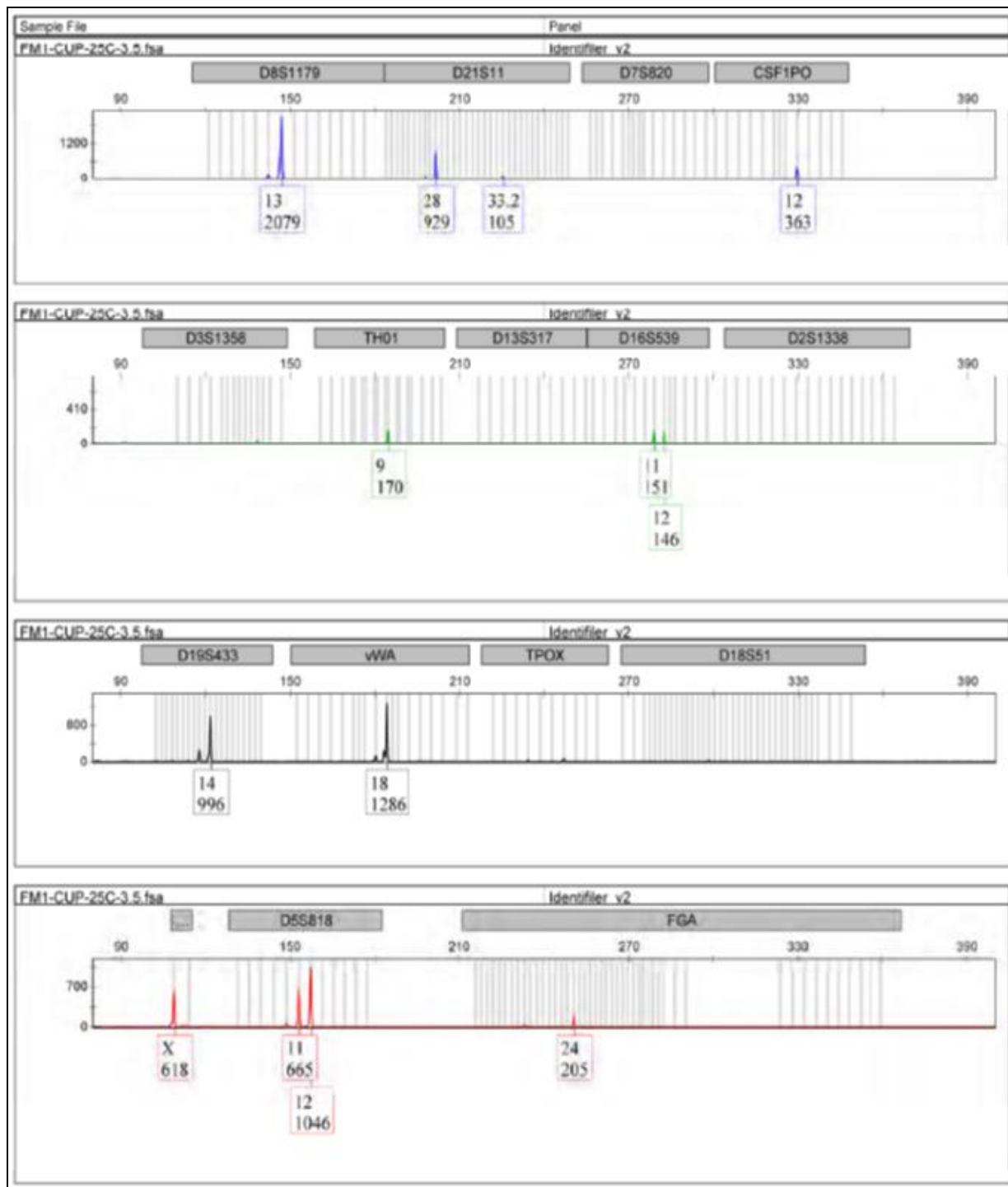
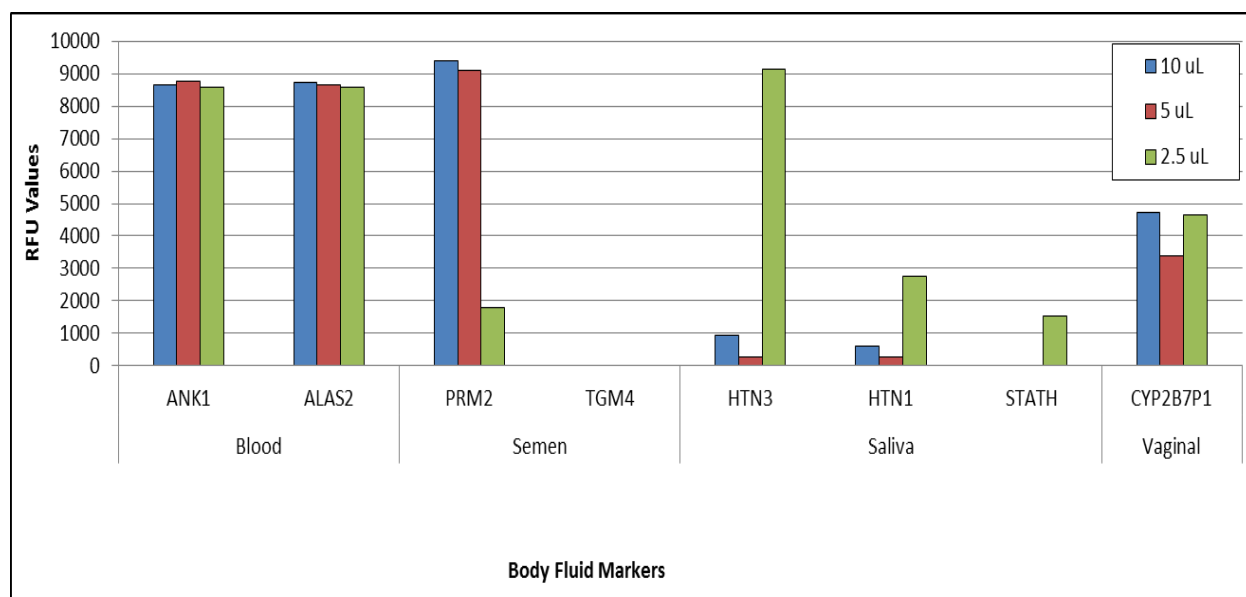


Figure 20: Profile obtained from coffee cup grip with 3.5 μ L reaction volume

RNA Results

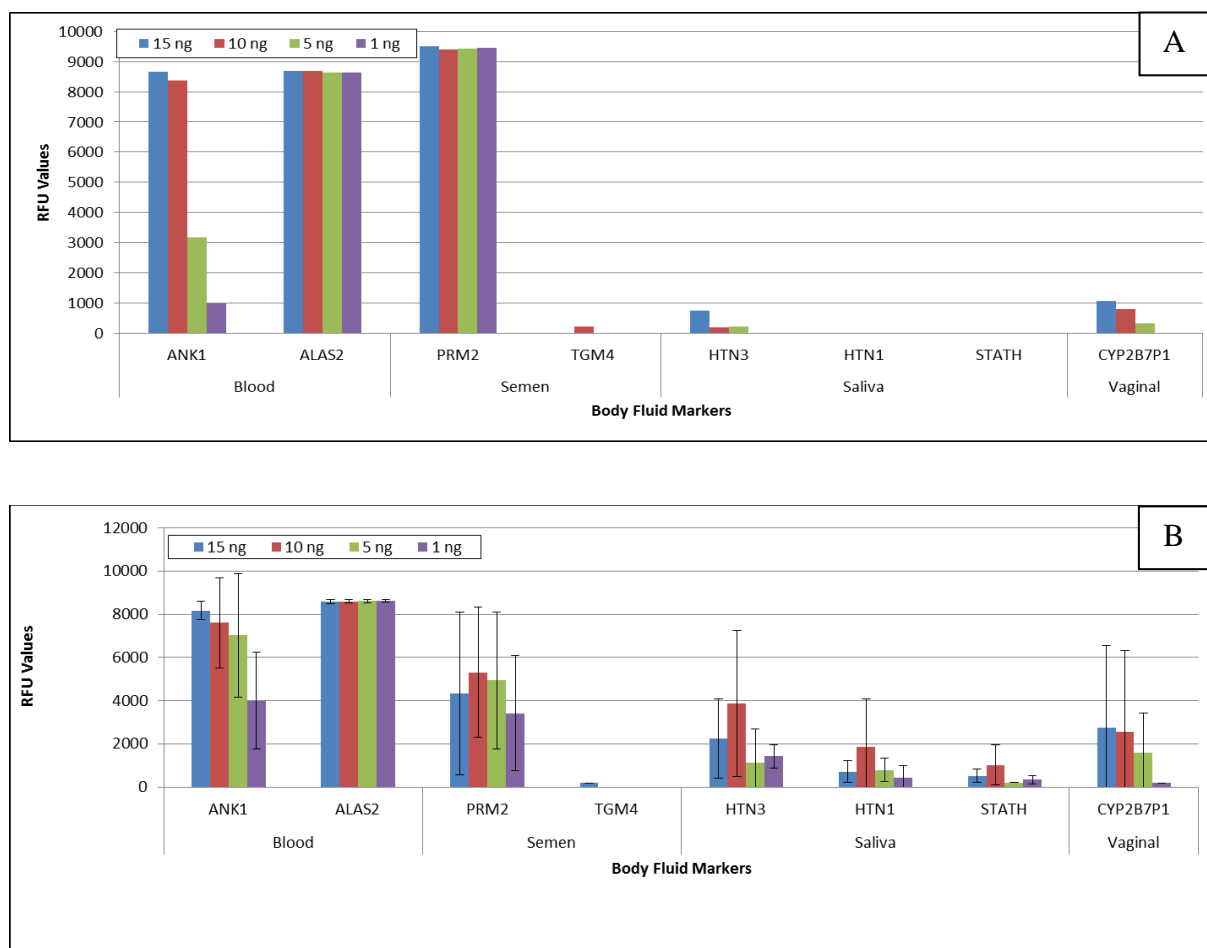
Due to two amplification sections during RNA analysis, the first experiment was aimed at only reducing the RT-PCR volume (reverse transcriptase treatment) and noting any observable patterns. Only four body fluids were tested: blood, vaginal, saliva, and semen. The RNA input was held at the standard 25 ng. The RT-PCR volumes tested were 10 μ L, 5 μ L, and 2.5 μ L (standard being 20 μ L). The body fluid multiplex volume was held at standard 25 μ L. Figure 21 shows that there is no observable pattern between the volumes, some markers had similar RFU values and others had higher RFU values with one volume(s) over the other(s).



(N=1 donor per body fluid) Comparison of RFU values between three different RT-PCR volumes while MP volume is standard

Figure 21: Comparison of RFU values at reduced RT-PCR volumes

Like with DNA, the goal was to optimize a methodology that can successfully identify tissue of origin at the few cells or single cell level. The next step was to decrease the RNA input and apply reduced RT-PCR volumes to determine if there is an advantage or pattern. The volumes tested were 5 μ L and 2.5 μ L. The RNA inputs used were 15 ng, 10 ng, 5 ng, and 1 ng. The MP amplification volume was held at standard 25 μ L.



(N=1 donor per fluid at 5 μ L, 4 donors per fluid at 2.5 μ L) Comparison of RFU values with decreasing RNA inputs at (A) 5 μ L and (B) 2.5 μ L RT-PCR volume

Figure 22: Comparison of RFU values at multiple RNA inputs at reduced RT-PCR volumes

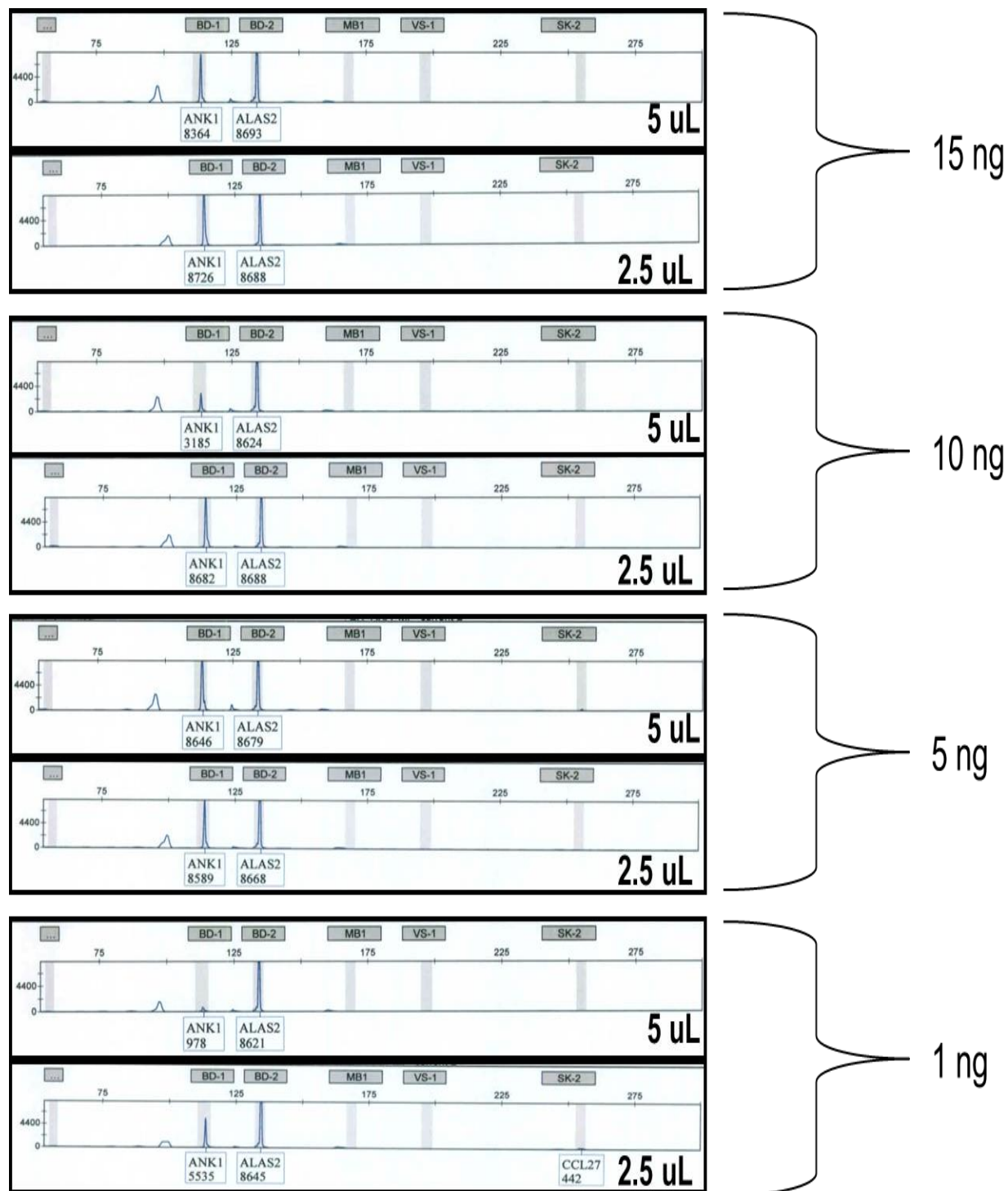
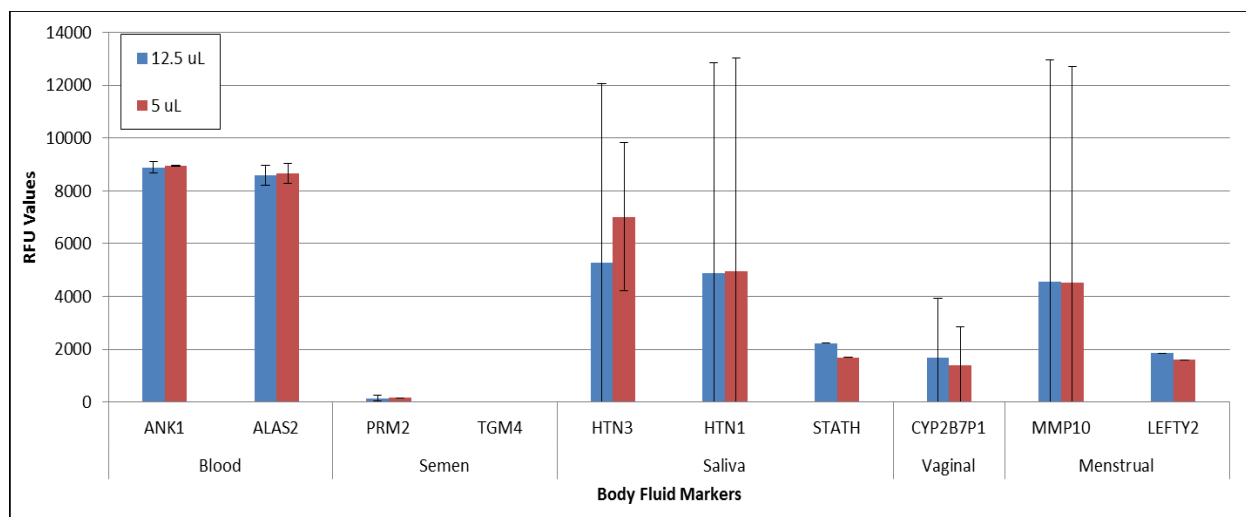


Figure 23: Profile comparisons of multiple RNA inputs at 5 μ L and 2.5 μ L RT-PCR volumes and standard MP amp volume

The results in Figures 22 and 23 show that there is no major discernible pattern with RFU values when decreasing RNA input and RT-PCR volume only. When looking at the graphs of Figure 22, it appears that there may be a possibility that reducing the RT-PCR volume may offer a small advantage for a few body fluid markers, but it seems that when applied alone, the results aren't strong enough to make this claim. Reducing the body fluid multiplex may be helpful for this issue but needed to be evaluated first.

Like with RT-PCR, the MP amp needs to be evaluated on its own first. The RT-PCR volume will be held at the standard 20 μ L. The RNA input will be held at the standard 25 ng as well. The MP amp volume will be tested at 12.5 μ L and 5 μ L.

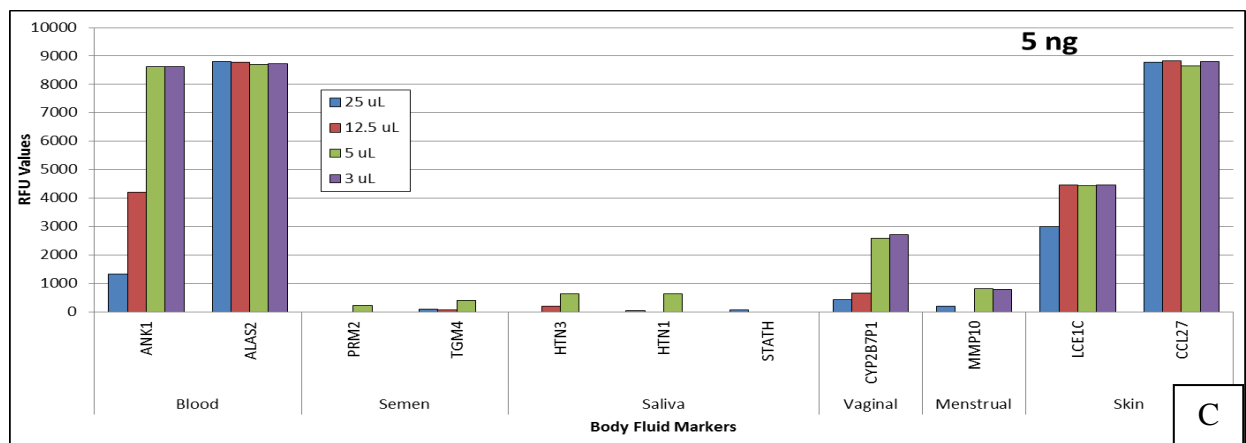
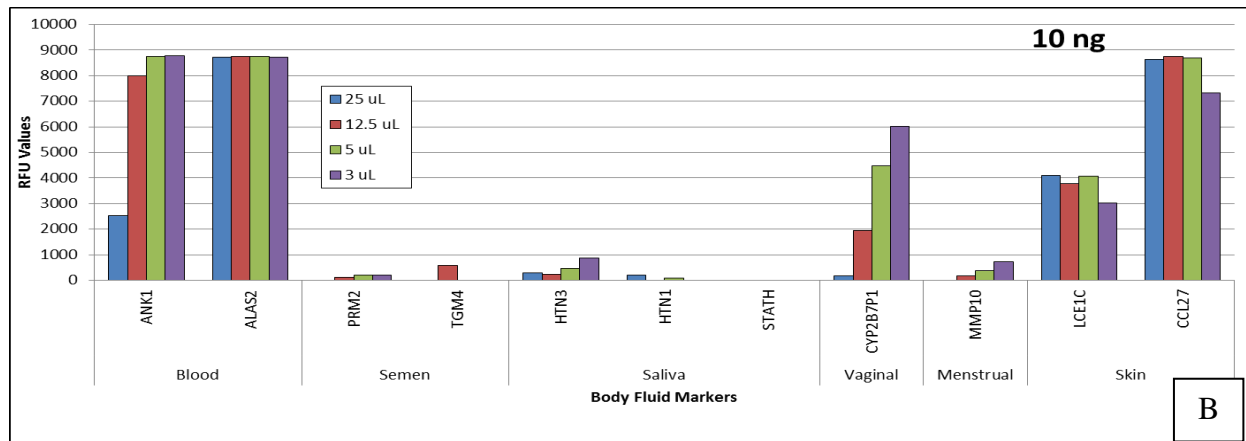
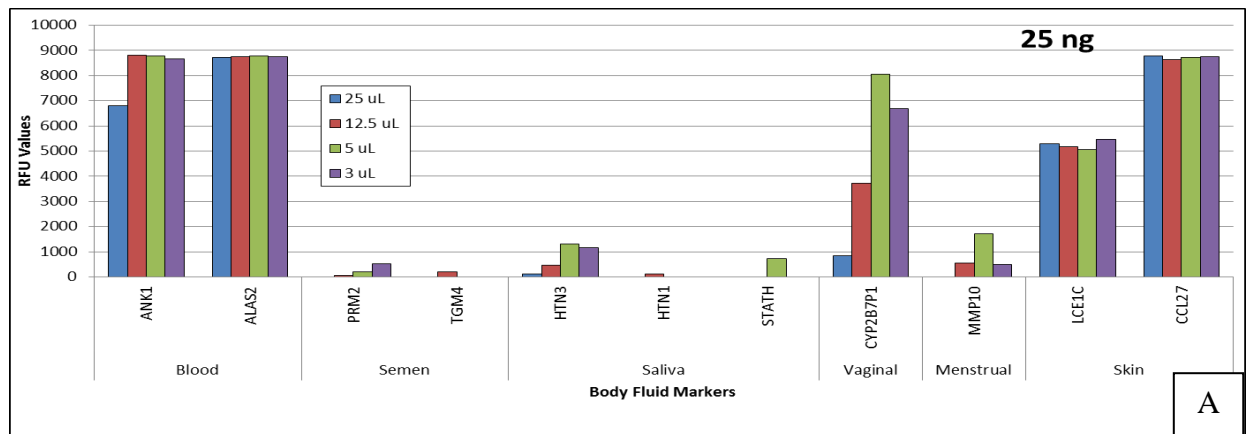


(N=2 donors per fluid) Comparison of RFU values at two reduced MP amp volumes while RT-PCR is held at standard

Figure 24: Comparison of RFU values for reduced MP volumes

At 25 ng, in Figure 24, there is no major difference between the two MP volumes. Variation of values at some of the markers was high, but may be contributed to the small sample set. At this point, like with RT-PCR, it is important to again decrease to RNA input and see if the reduced MP volume will have any effect on the results. The RT-PCR volume was held at standard 20 μ L as the RNA inputs tested are 25 ng, 10 ng, and 5 ng. The MP volumes used are 25 μ L, 12.5 μ L, 5 μ L, and 2.5 μ L.

As observed in Figure 25, as the MP amp volume is reduced, there is a tendency for increased sensitivity which in turn produces higher RFU values. There also appears to be an improvement in detection as some body fluid markers which were not detected at standard levels, were only detected when using reduced volume. Figure 26 shows several profiles of a vaginal sample at 25 ng. At the standard volume, the RFU value is low, under 1000. As the volume decreases, the RFU values increases, with the highest value obtained at 5 μ L.



(N=1 donor per body fluid) Comparison of RFU values for multiple MP amp volumes with decreasing RNA inputs of (A) 25 ng, standard, (B) 10 ng, and (C) 5 ng

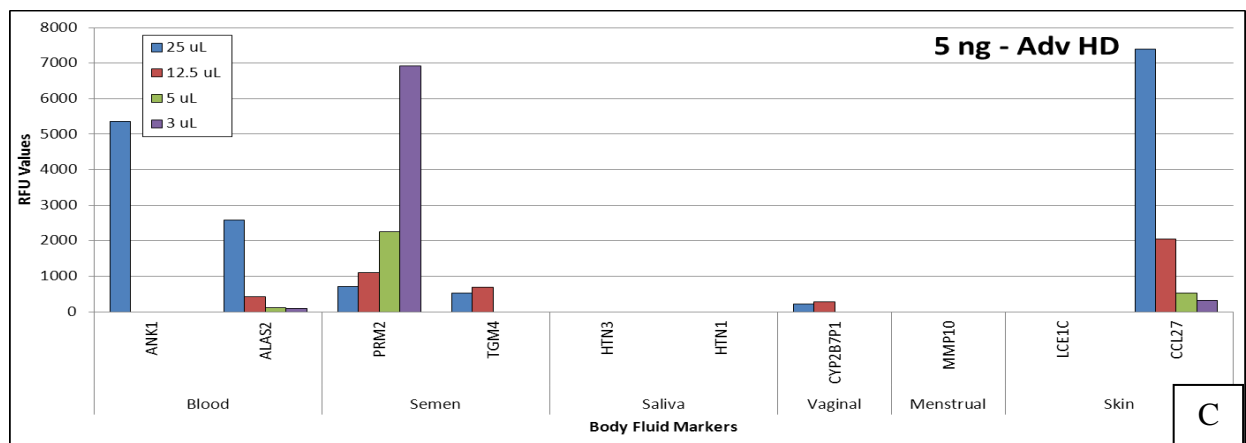
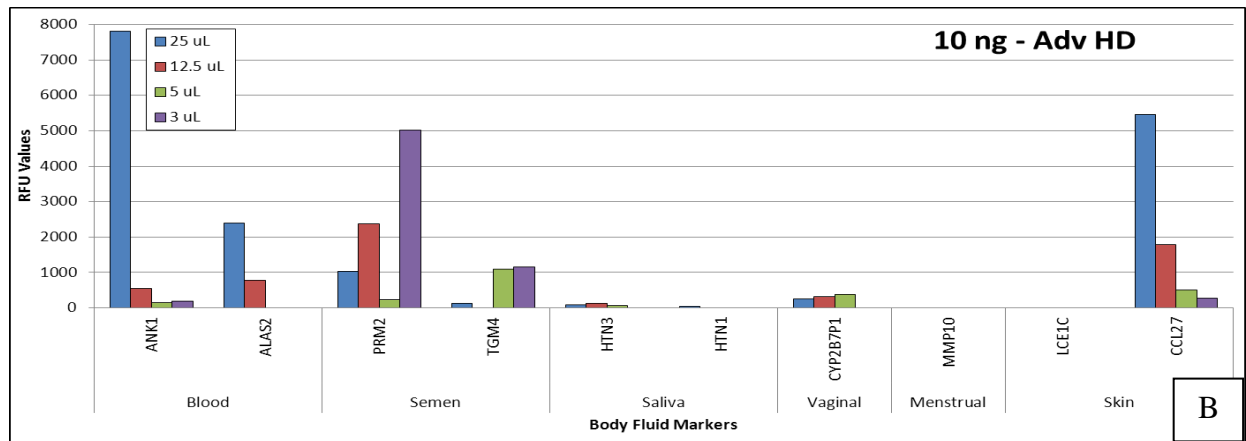
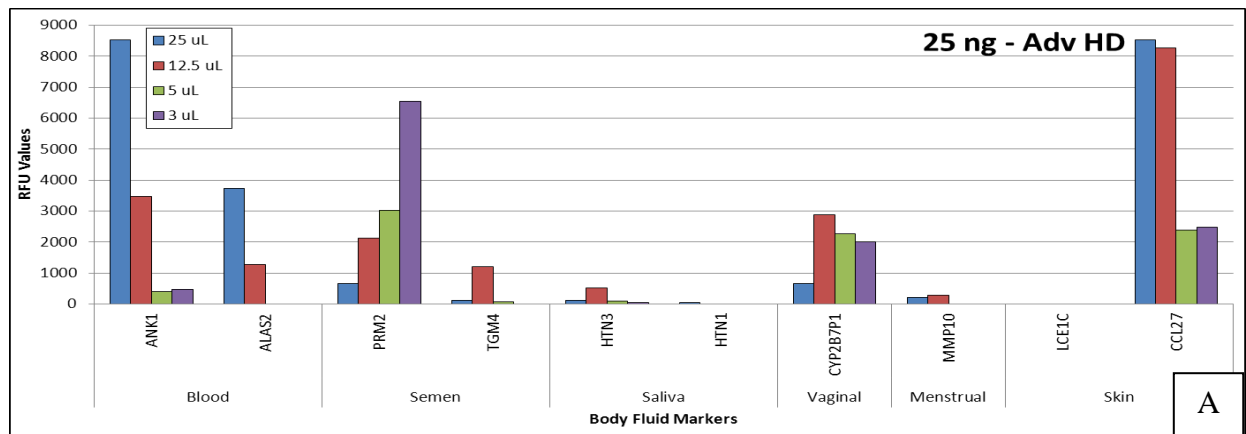
Figure 25: Comparison of RFU values for decreasing RNA inputs for reduced MP amp volumes



Figure 26: Profiles of a vaginal sample at 25 ng with decreasing MP amp volume

Another multiplex kit was available for use. This kit was the Adv. HD Multiplex kit. With it come several advantages as stated by the manufacturer such as a reduced amp program by about two hours, increased sensitivity, increased efficiency, and the STATH primer was removed from the primer mix. The program for this kit is slightly different: 98°C for 2 minutes, 35 cycles of 94°C for 10 seconds, 55°C 10 seconds (+0.2°C per cycle), and 72°C for 20 seconds, then 72°C for 5 minutes ended by a 4°C hold. The RT-PCR volume was held at standard 20 µL as the RNA inputs tested are 25 ng, 10 ng, and 5 ng. The MP volumes used are 25 µL, 12.5 µL, 5 µL, and 2.5 µL.

Shown in Figure 27, the results were much different than what was observed with standard MP amp kit. With the standard MP amp kit, a pattern was observed in which there was a tendency for increased detection and sensitivity at lower volumes. With the Adv. HD kit, there seems to be no observable pattern. A few markers have higher sensitivity with higher volumes and then other markers are observed to have the opposite pattern. When in the process of developing an optimal protocol, a pattern that shows benefit of methods needs to be observed. In this case, utilization of the Adv. HD MP amp kit did not show any clear patterns of advantage and overall benefit and was not used in subsequent experiments.

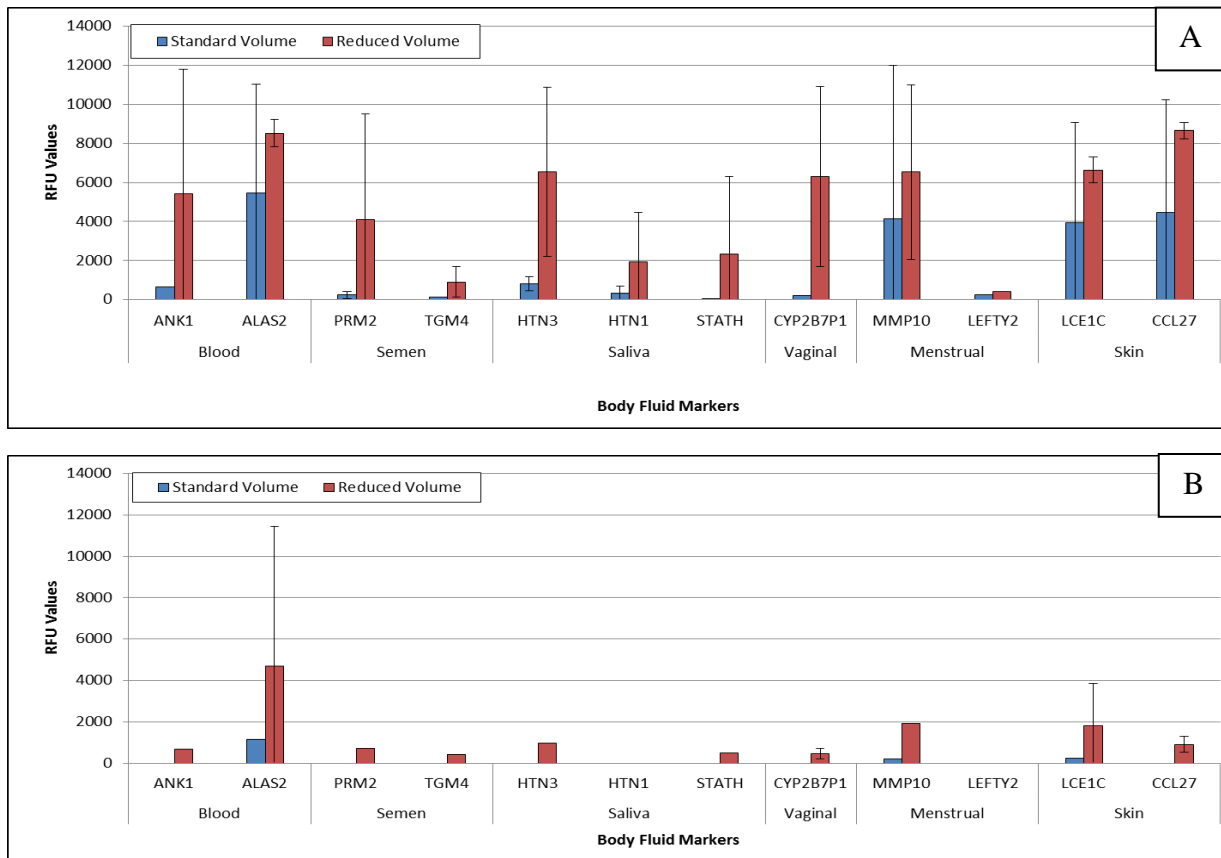


(N=1 donor per body fluid) Comparison of RFU values for multiple Adv HD MP amp volumes with decreasing RNA inputs of (A) 25 ng, standard, (B) 10 ng, and (C) 5 ng

Figure 27: Comparison of RFU values of the Adv HD MP amp kit with reduced RNA inputs and MP volume

It was decided that the best MP amp volume to work with was 5 μ L. It provided the highest sensitivity among all of the reduced volumes. Since by this point both amps involved in RNA analysis had been evaluated, reduced volume of both parts were combined to determine if there was any benefit to being fully reduced volume. For the next experiment, the RT-PCR amp and the MP amp volumes were tested together at 5 μ L. The samples used were also tested with both amps at standard volumes for accurate comparison. More RNA inputs were tested with these experiments. Aside from the 10 ng, 5 ng, and 1 ng inputs tested previously, inputs of 0.5 ng, 0.25 ng, 0.1 ng, and 0.05 ng were added.

Figure 28 displays the results from the reduced volume amps at 1 ng and 0.05 ng. Decreasing towards the 1 ng input, most body fluid markers tended to show an increase in sensitivity, even if slightly, with reduced volume amps. The 1 ng input is when there was first an increase in sensitivity for every body fluid marker. With the exception of the menstrual marker LEFTY2, in which the increase is very slight, the remaining body fluid markers show a vast increase in RFU values over standard volumes. At the lowest input tested, 0.05 ng, not only was an increase in sensitivity observed, but there was also a major increase in detection of body fluid markers that weren't previously detected with standard volumes.

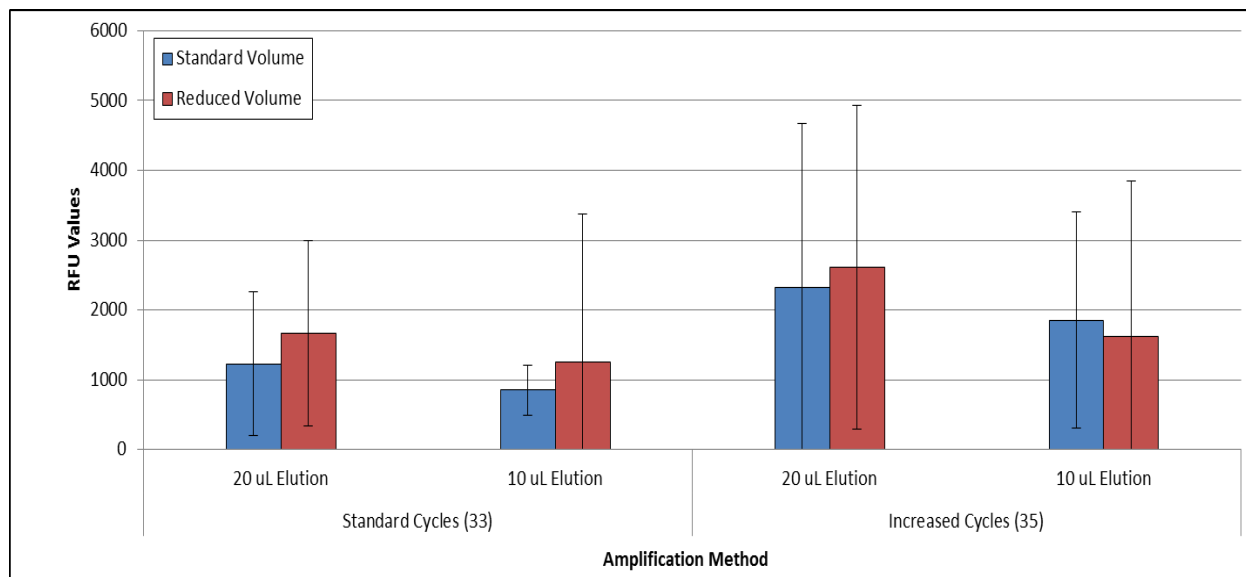


(N=2 donors per body fluid) Comparison of RFU values for standard and reduced RT-PCR/MP amp volumes at (A) 1 ng and (B) 0.05 ng

Figure 28: Comparison of RFU values with reduced RNA inputs at 5 μ L RT-PCR and MP amp volumes

As input is decreasing in amount, it is important to apply this developed method towards realistic touch samples collected via swab. Different “touched” surfaces were swabbed, as skin samples, and extracted following standard RNA extraction. Two elution volumes were compared, 20 μ L (standard) and 10 μ L to determine if quality would be increased. These samples were also tested with increased cycles (35) and at standard and reduced (5 μ L) RT-PCR and multiplex volumes.

When comparing only elution volumes, 10 μ L did not display an increase in RFU values over 20 μ L. Using this elution volume as an option was discarded as 20 μ L still provided ideal results. When comparing only standard and increased cycles, it is reasonably understood that with increased cycle number, RFU values were increased as well. Although this is the case, standard cycles still provide acceptable results (around 1000 RFUs). When comparing standard and reduced reaction volumes, ideally looking at the 20 μ L elution volume as that is what was more ideal to use, reduced volume exhibited an increase in sensitivity over standard (Figure 29).



(N=5 touch samples per elution volume) Comparison of RFU values for standard and reduced RT-PCR/MP amp volumes with standard and increased cycle numbers and 20 µL and 10 µL extraction elution volumes.

Figure 29: Comparison of RFU values for multiple extraction elution volumes and standard and increased cycle numbers, all at the 5 µL RT-PCR/MP amp volume.

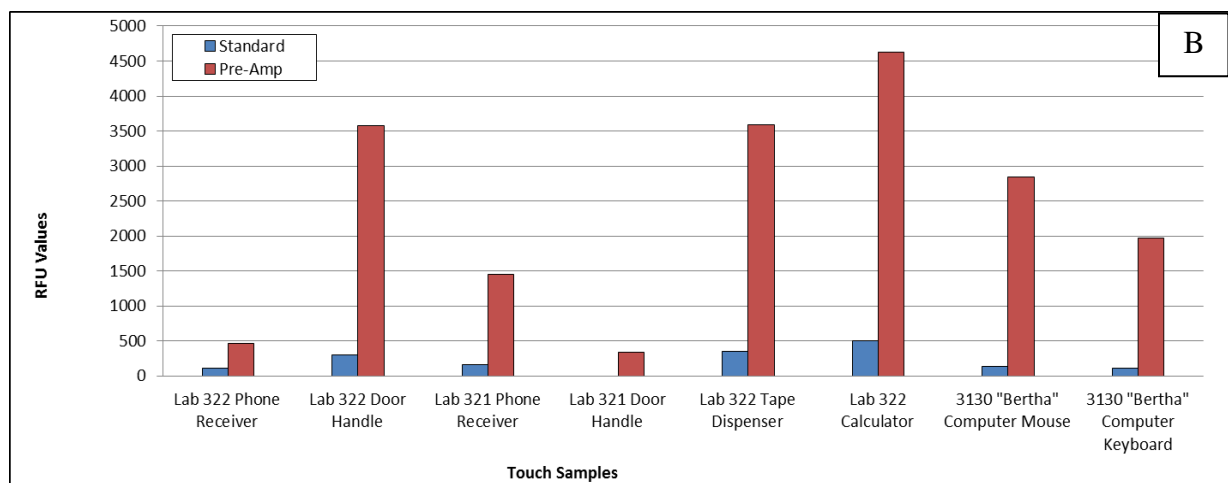
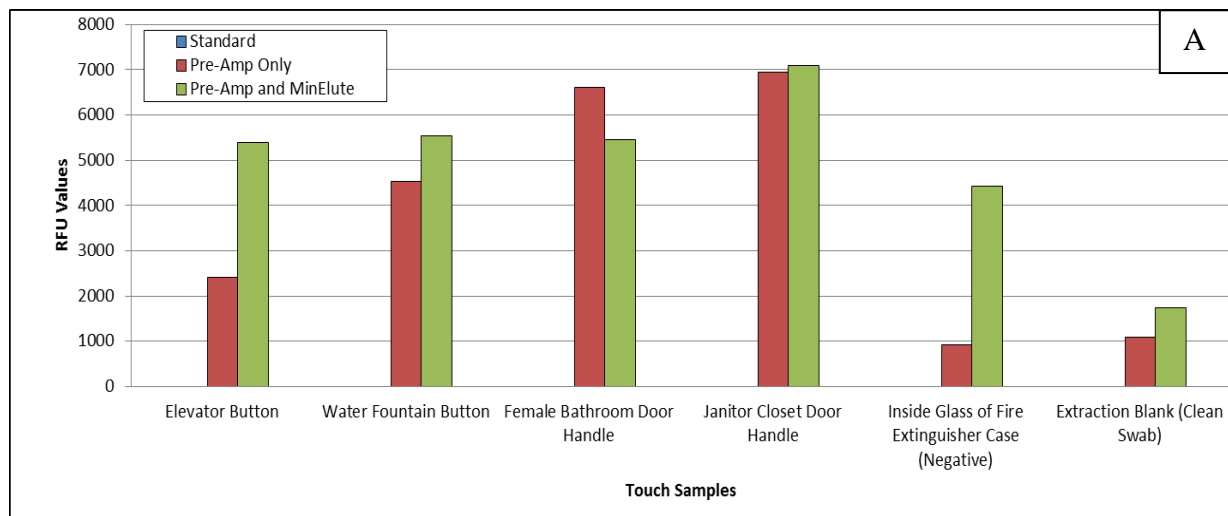
Saliva and vaginal samples would also be forensically relevant samples found at trace levels. Unlike skin samples, in which swabs are collected of touched surfaces, saliva and vaginal sample cells are counted via hemacytometer following the protocol describe in RNA methodology. These samples were tested with standard and reduced RT-PCR and multiplex amp volumes. Table 2 shows the results from the cell counts. The lowest count with detection was around about five cells. Unfortunately, there was no developed pattern as the detection tended to appear random. Due to inaccuracies of the hemacytometer, this method was discarded and alternate methods to increase detection and sensitivity for touch samples were investigated.

Table 2: Comparison of saliva (SA) and vaginal (VS) cell count samples for standard and reduced volumes. A checkmark indicates detection of at least one body fluid specific marker at that particular cell count. A gray box before first checkmark indicates that the cell count started lower.

	Cell Averages (cells/uL) – Standard Volumes									
Samples	>1000	~750	~500	~250	~100	~50	~25	~15	~5	~1
SA17	✓	✓	✓							
SA18	✓	✓	✓	✓	✓	✓	✓	✓		
SA19				✓						
VS1a										
VS24										
VS25		✓	✓	✓						

	Cell Averages (cells/uL) – Reduced Volumes									
Samples	>1000	~750	~500	~250	~100	~50	~25	~15	~5	~1
SA17	✓	✓	✓	✓						
SA18	✓	✓	✓	✓	✓	✓	✓	✓	✓	
SA19										
VS1a										
VS24										
VS25										

Another possible advantage to use with single and few cells is preamplification. As explained in RNA methodologies, the preamplification step occurs before the MP amp, only using the LCE1C skin specific primer, to increase the chances of detection of skin in touch swab samples. Two sets of multiple swabs were taken with one set going from preamplification to the MP amp, and the second set added a MinElute purification step before the MP amp.



Comparison of RFU values for (A) standard, pre-amp, and pre-amp with MinElute analysis methods with standard reaction volumes and (B) Further validation of pre-amp only.

Figure 30: Comparison of RFU values of multiple pre-amplification methods applied to touch samples

Figure 30 shows the results from the preamplification experiments. Following standard RNA analysis, skin was not detected in any of the samples. When comparing the two preamplification sets, with MinElute, RFU values tended to be higher, but as seen for

preamplification only in both (A) and (B), preamplification alone still provides ideal results and will save time and materials over MinElute. The issue that appeared with both experiments was the detection of skin in blank samples. It was a concern that the preamplification step may be too sensitive and cannot be reliable. In order to investigate this further, and to proceed to the ultimate goal of this project, particles would need to be collected and tested with preamplification, along with multiple blank samples.

Following the collection procedure mentioned for micro-manipulation, particles were collected from collected skin slides. Multiple locations were used as donors and particles were collected in groups of 10, 25, 50, 100, and 0 (as a negative area collection). Clumps, groups of multiple particles, were collected as well in groups of 1, 5, 10, 20, and 0 (as a negative area collection). For these experiments, 5 μ L of sample was used in the preamplification step and 5 μ L of that product was used in the MP amp. The number of cycles of preamplification was increased to 14. Only the RT-PCR volume was reduced to 5 μ L and MP amp volume was kept standard.

Table 3 shows the results from the particle and clump experiments. There was good detection of skin down to 10 particles and even in 1 clump. Detection in the negative area samples could be due to accidental pick up during collection, but every blank sample added did not have detection of any body fluid marker, showing that preamplification can be reliable. Also, the clump samples were tested without the preamplification step, for comparison, with no detection of any body fluids.

Table 3: Comparison of LEC1C RFU values from collected particles and clumps with the preamplification step at reduce RT-PCR volume (5 μ L) and standard MP volume.

	Particles Collected (RFU Values of LCE1C Peaks)				
Donors	0	10	25	50	100
Shirt Collar		3142			
Lab Coat			6881		
Sock	3648		5703		
Shirt Back		4085			
Sweater Sleeve	3128				

	Clumps Collected (RFU Values of LCE1C Peaks)				
Donors	0	1	5	10	20
Shirt Collar		5806	3097	2480	2644
Jean Pant Leg				2200	
Sock					3463
Shirt Back	3220	2969			
Sweater Sleeve					

Several more experiments were carried out for particles and clumps. Preamplification cycles were increase to 20 to see if there was an increase in detection or in sensitivity. These results showed no advantage with increased cycles, so reduced MP amp volumes was tested as well. For this experiment, 3 μ L of preamplification product was added into 7 μ L of MP amp master mix (total volume of 10 μ L). The results showed the same detection patterns with no increase in sensitivity.

CHAPTER FOUR: CONCLUSION

The goal of this research was to develop an optimal DNA and RNA assay that could determine identification and tissue source origin of trace samples that could even be applied to the analysis of individually collected cells. Traditionally, trace samples have been analyzed using standard methods, but due to poor profile quality, methods that are usually employed for low copy number samples have been applied to trace analysis. Although these methods have shown some success, they tend to be laborious, requiring a lot of extra time and materials, and may require extra training beyond the standard analysis method. Along with these issues, although these methods have been examined at single cell equivalents, none of these studies have actually collected individual cells from trace samples and applied their methods. This study aimed to optimize the reduced volume method to the micro volume method and has applied this method to actually collected cells from trace samples. Within this study, multiple micro volumes were examined to determine the most ideal at single cell DNA equivalents, which showed a 50% profile recovery without adding additional method aside from reducing the amplification volume. When applied to collected cells, using the reduced volume, the same profile recovery rate was observed, with almost 80% observed for two cells. With RNA, detection was observed at the 0.05 ng level, a fraction of the 25 ng amount used in standard analysis. With collect skin particles, using a reduced RT-PCR volume and with additional aid of a preamplification step, detection of skin could be obtained from as few as ten particles and even in one clump.

The first micro volume extensively studied was the 1 μL reaction volume. Due to concerns over evaporation, addition methods of mineral oil overlays, sealing solution overlays, and sealing solution with MinElute post-amplification purification was applied. Even though sealing solution only provided ideal results, it was too difficult to recover the sample from the overlay, and additional post-amplification methods had to be applied. NucleoSpin purification, with an elution volume of 3 μL , was a viable choice as it provided high profile recovery and RFU values. However, it was thought that there was still a way to obtain ideal results without the use of an additional purification step.

Other micro volumes were evaluated, with 2.5 μL first being investigated as a possible option, but exhibited high rates of evaporation which were possibly hindering higher profile recovery rates. Through further testing of micro volumes, 3.5 μL was found to be the most ideal as it had the highest profile recovery and lowest evaporation. Along the way, the results were compared at increased PCR cycles. As expected, RFU values were much higher, but there was also an increase in stochastic effects. When looking at profile recovery, standard and increased cycle numbers tended to be very similar, and at 3.5 μL , standard had a higher profile recovery among all of the increased cycle sets. For individual cell collection, cells were placed directly into the 3.5 μL amplification mix. These experiments were also compared to standard volume. With the observed results, it is clear that the micro volume is an ideal method as it provided higher profile recovery.

For individual cells, though, it was found necessary that PCR cycles needed to be increased, as the cells were not extracted before application to the micro volume reaction mix and thus are more difficult to work with than DNA equivalents. With just one cell, when

applying the 3.5 μL volume, about 40% of a profile was recovered. Only about 20% was recovered using standard volume. At two cells, 80% of a profile was recovered at the 3.5 μL volume as opposed to 70% from standard volume. Although this is only a 10% difference, the 10% gained from reduced volume could be what is needed to identify the individual. These DNA experiments have shown that this is the optimal assay for identification for trace samples as it doesn't require any addition steps, provided ideal results without additional methods, aside from increase in cycle number for single cells, has minimal evaporation rates, cuts costs by using less reagents, and has proven that it can be applied to individual cells and still obtain nearly half of a profile.

There were several methods evaluated for RNA analysis as well. First, the RT-PCR step was evaluated at reduced volume levels. No additional methods were applied, as there was no observable pattern formed by reducing the volume. More evaluation was used when studying the body fluid multiplex amplification. At first, the standard MP kit was reduced to multiple volumes. The 5 μL volume provided the highest level of sensitivity, as any lower started experiencing issues with marker drop out. Another MP kit, the Adv HD MP kit, became a possible option as it was designed to have a shorter amplification time with increased sensitivity and efficiency. When compared to the standard MP kit, it had lower sensitivity and tended to experience a higher level of drop out at the reduced volume and reduced RNA input levels. This is most likely due to the fact that it was optimized for a higher volume and input and thus does not perform as ideally at lower levels.

With this method discarded as an option, this study proceeded in evaluating reduced RT-PCR and MP amp volumes at 5 μL each. At the lowest input tested, 0.05 ng, sensitivity was

much greater than at standard volumes, not to mention increase in detection as some body fluid markers were only detected with the reduced volumes. Seen as the most ideal protocol to apply to touch skin samples, it was found that an additional method was needed. Preamplification of one of the skin markers was evaluated as a useful tool in combination with reduced volume to increase detection of the LCE1C marker. By applying preamplification before the MP amp, skin was detected in touch samples whereas there was no detection previously. However, an issue came up with detection in some of the blank samples. It was called into question of whether preamplification was too sensitive and would amplify anything. These issues were dismissed when preamplification was applied to single particle and clump collection. Different areas of collection were evaluated, such as the inside of a sock and the sleeve of a sweater, and particles and clumps were collected from each. They were immediately placed into a 5 μ L RT-PCR volume, and for clumps, additional collections were made for standard volume evaluation. The preamplification method was used with increased cycles, for the same reason of DNA cell evaluation, and the MP volume was kept at standard. Skin detection was obtained down to 10 particles and down to even a single clump of particles. There was detection of skin in the negative area samples, but as mentioned previously, this could be due to accidental pick up. The blank samples had no detection of LCE1C. At standard volume, nothing was detected in any of the clumps. When this same experiment was repeated with reduced MP volume, detection was identical, although RFU values were lower, unlike what has been previously seen. This could be due to the preamplification step amplifying samples so much that there is some inhibition when the next amp uses less volume, not having enough room for the amp to take place. So, although the RNA assay doesn't utilize reduced volume throughout the entire process, the reduced RT-

PCR will still save reagent and, along with the preamplification step, can successfully determine whether a sample is of skin origin down to a small amount of collected particles.

Through this study, two optimized assays were developed that provided ideal results for identification and determination of body fluid down to single cell or few cell levels. As previously mentioned, there have been numerous studies aimed at trace samples and improved analysis, but none have actually removed cells from collected trace samples and applied their developed methods, and that is exactly what this study did. This study was able to prove that by developing an optimized and robust method for analysis of single and few cell equivalents, it is possible to get the same results when applying this same method to individual cells. This is ground breaking in that this displays the very near possibilities of eliminating mixtures in trace samples and the ability to obtain identity and body tissue origin from just a few cells or even just one single cell. At this rate, it will begin to become nearly impossible to get away with a crime.

There is still more work that needs to be done is currently underway. Being able to obtain the results that were found in this study is unique in itself, but there are more studies being done to improve the profile recovery rates even further. One study currently being conducted is the investigation of determining the most ideal areas to collect trace samples from different materials. For example, if a victim was physically assaulted, it may be more idea to collect a trace sample slide from one area of their skin over another because that area may shed less cells, thus contributing less to a mixed trace sample. Once the trace samples are collected, as the results displayed, sometimes profiles or skin detection were obtained with just one cell/particle, and sometimes more. Current studies are also underway to examine different morphological characteristics of particles on trace samples. These characteristics will be compared between the

collected particles to determine which physical characteristics may lead to a higher chance of obtaining a profile or detection of skin. By utilizing the developed assays of this study, in combination with the current/future studies, it may be possible to one day obtain full profiles from just one cell, and be able to identify its tissue origin every time. The results are very promising towards making this overall goal of the field of forensic biology a not-too-distant reality.

REFERENCES

- [1] McDonald, Jessica, and Donald Lehman. "Forensic DNA Analysis." *Focus: Forensic Science*. 25.2 (2012): 109-13. Print.
- [2] Carey, Loucinda, and Luba Mitnik. "Trends in DNA forensic analysis." *Electrophoresis*. 23. (2002): 1386-97. Print.
- [3] Schneider, Peter. "Basic issues in forensic DNA typing." *Forensic Science International*. (1997):17-22. Print.
- [4] Jeffreys, Alec, Victoria Wilson, et al. "DNA "Fingerprints" and Segregation Analysis of Multiple Markers in Human Pedigrees." *American Journal of Human Genetics*. (1986): 11-24. Print.
- [5] Masters, John, Jim Thomson, et al. "Short tandem repeat profiling provides an international reference standard for human cell lines." *PNAS*. 98.14 (2001): 8012-17. Print.
- [6] Moretti, Tamyra, Anne Baumstark, et al. "Validation of Short Tandem Repeats (STRs) for Forensic Usage: Performance Testing of Fluorescent Multiplex STR Systems and Analysis of Authentic and Simulated Forensic Samples." *Journal of Forensic Science*. 46.3 (2001): 647-60. Print.
- [7] Butler, John, Eric Buel, et al. "Forensic DNA typing by capillary electrophoresis using the ABI Prism 310 and 3100 genetic analyzers for STR analysis." *Electrophoresis*. 25. (2004): 1397-1412. Print.
- [8] Kimpton, Colin, Petere Gill, et al. "Automated DNA profiling employing multiplex amplification of short tandem repeat loci." *PCR Methods and Applications*. 3. (1993): 13-22. Print.
- [9] Kimpton, Colin, Deborah Fisher, et al. "Evaluation of an automated DNA profiling system

- employing multiplex amplification of four tetrameric STR loci." *International Journal of Legal Medicine*. 106.6 (1994): 302-11. Print.
- [10] Hammond, Holly, Li Jin, et al. "Evaluation of 13 short tandem repeat loci for use in personal identification applications." *American Journal of Human Genetics*. 55. (1994): 175-89. Print.
- [11] Butler, John. "Genetics and genomics of core short tandem repeat loci used in human identity testing." *Journal of Forensic Science*. (2006). Print.
- [12] Juusola, Jane, and Jack Ballantyne. "Messenger RNA profiling: a prototype method to supplant conventional methods for body fluid identification." *Forensic Science International*. (2003): 85-96. Print.
- [13] Juusola, Jane, and Jack Ballantyne. "Multiplex mRNA profiling for the identification of body fluids." *Forensic Science International*. 152.1 (2005): 1-12. Print.
- [14] Bauer, M., and D. Patzelt. "Protamine mRNA as molecular marker for spermatozoa in semen stains." *International Journal of Legal Medicine*. 117.3 (2003): 175-79. Print.
- [15] Sakurada, Koichi, Hiroshi Ikegaya, et al. "Evaluation of mRNA-based approach for identification of saliva and semen." *Legal Medicine*. 11.3 (2009): 125-128. Print.
- [16] Fleming, Rachel, SallyAnn Harbison, et al. "The development of a mRNA multiplex RT PCR assay for the definitive identification of body fluids." *Forensic Science International: Genetics*. 4.4 (2010): 244-56. Print.
- [17] Visser, Mijke, Dmitry Zubakov, et al. "mRNA-based skin identification for forensic applications." *International Journal of Legal Medicine*. 125.2 (2011): 253-63. Print.
- [18] Haas, C., E. Hanson, et al. "Selection of highly specific and sensitive mRNA biomarkers for the identification of blood." *Forensic Science International: Genetics*. 5.5 (2011): 449-58. Print.

- [19] Fang, R., C.F. Manohar, et al. "Real-time PCR assays for the detection of tissue and body fluid specific mRNAs." *International Congress Series*. 1288. (2006): 685-7. Print.
- [20] Juusola, Jane, and Jack Ballantyne. "mRNA Profiling for Body Fluid Identification by Multiplex Quantitative RT-PCR." *Journal of Forensic Science*. 52.6 (2007): 1252-62. Print.
- [21] Haas, C., B. Klessner, et al. "mRNA profiling for body fluid identification by reverse transcription endpoint PCR and realtime PCR." *Forensic Science International: Genetics*. 3.2 (2009): 80-8. Print.
- [22] Noreault-Conti, Trisha, and Eric Buel. "The Use of Real-Time PCR for Forensic Stain Identification." *Profiles in DNA*. (2007): 3-5. Print.
- [23] Hanson, E., and J. Ballantyne. "Highly specific mRNA biomarkers for the identification of vaginal secretions in sexual assault investigations." *Science & Justice*. 53.1 (2013): 14-22. Print.
- [24] Roeder, Amy, and Cordula Haas. "mRNA profiling using a minimum of five mRNA markers per body fluid and a novel scoring method for body fluid identification." *International Journal of Legal Medicine*. 127. (2013): 707-21. Print.
- [25] Hanson, E., C. Haas, et al. "Specific and sensitive mRNA biomarkers for the identification of skin in 'touch DNA' evidence." *Forensic Science International: Genetics*. 6.5 (2012): 548-58. Print.
- [26] Hanson, E., C. Haas, et al. "Identification of skin in touch/contact forensic samples by messenger RNA profiling." *Forensic Science International: Genetics Supplement Series*. 3.1 (2011): 305-6. Print.
- [27] van Oorschot, Roland, Kaye Ballantyne, and R. John Mitchell. "Forensic trace DNA: a review." *Investigative Genetics*. (2010). Print.

- [28] Bright, Jo-Anne, and Susan Petricevic. "Recovery of trace DNA and its application to DNA profiling of shoe insoles." *Forensic Science International*. 145.1 (2004): 7-12. Print.
- [29] Sewell, Jonathan, Ignacio Quinones, et al. "Recovery of DNA and fingerprints from touched documents." *Forensic Science International: Genetics*. 2.4 (2008): 281-5. Print.
- [30] Petricevic, Susan, Jo-Anne Bright, and Sarah Cockerton. "DNA profiling of trace DNA recovered from bedding." *Forensic Science International*. 159.1 (2006): 21-6. Print.
- [31] Pizzamiglio, M., A. Mameli, et al. "Forensic identification of a murderer by LCN DNA collected from the inside of the victim's car." *International Congress Series*. 1261. (2004): 437-9. Print.
- [32] Rutty, G.N. "An investigation into the transference and survivability of human DNA following simulated manual strangulation with consideration of the problem of third party contamination." *International Journal of Legal Medicine*. 116. (2002): 170-3. Print.
- [33] Prinz, Mechthild, Linnea Schiffner, et al. "Maximization of STR DNA typing success for touched objects." *International Congress Series*. 1288. (2006): 651-3. Print.
- [34] Pang, B.C.M., and B.K.K Cheung. "Double swab technique for collecting touched evidence." *Legal Medicine*. 9.4 (2007): 181-4. Print.
- [35] van Oorschot, R.A.H., D.G. Phelan, et al. "Are you collecting all the available DNA from touched objects?." *International Congress Series*. 1239. (2003): 803-7. Print.
- [36] Hansson, Oskar, Marianne Finnebraaten, et al. "Trace DNA collection—Performance of minitape and three different swabs." *Forensic Science International: Genetics Supplement Series*. 2.1 (2009): 189-90. Print.
- [37] Gill, P., C.H. Brenner, et al. "DNA commission of the International Society of Forensic Genetics: Recommendations on the interpretation of mixtures." *Forensic Science International*. 160. (2006): 90-101. Print.

- [38] Dixon, L.A., A.E. Dobbins, et al. "Analysis of artificially degraded DNA using STRs and SNPs—results of a collaborative European (EDNAP) exercise." *Forensic Science International*. 164.1 (2006): 33-44. Print.
- [39] Holland, Mitchell, Christopher Cave, et al. "Development of a Quality, High Throughput DNA Analysis Procedure for Skeletal Samples to Assist with the Identification of Victims from the World Trade Center Attacks ." *Croatian Medical Journal*. 44.3 (2003): 264-72. Print.
- [40] Schumm, James, Robyn Wingrove, et al. "Robust STR multiplexes for challenging casework samples." *International Congress Series*. 1261. (2004): 547-9. Print.
- [41] Budowle, Bruce, Arthur Eisenberg, et al. "Low copy number typing has yet to achieve “general acceptance”." *Forensic Science International: Genetics Supplement Series*. 2.1 (2009): 551-2. Print.
- [42] Balding, David, and John Buckleton. "Interpreting low template DNA profiles." *Forensic Science International: Genetics*. 4. (2009): 1-10. Print.
- [43] Gill, Peter, Jonathan Whitaker, et al. "An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA." *Forensic Science International*. 112. (2000): 17-40. Print.
- [44] Ballantyne, Kaye, Roland van Oorschot, and R. John Mitchell. "Comparison of two whole genome amplification methods for STR genotyping of LCN and degraded DNA samples." *Forensic Science International*. 166.1 (2007): 35-41. Print.
- [45] Balogh, M.K., C. Børsting, et al. "Application of whole genome amplification for forensic analysis." *International Congress Series*. 1288. (2006): 725-7. Print.
- [46] Thacker, C.R., M.K. Balogh, et al. "The effect of whole genome amplification on samples originating from more than one donor." *International Congress Series*. 1288. (2006):

722-4. Print.

- [47] Pan, Xinghua, Alexander Urban, et al. "A procedure for highly specific, sensitive, and unbiased whole-genome amplification." *PNAS*. 105.40 (2008): 15499-504. Print.
- [48] Hanson, Erin, and Jack Ballantyne. "Whole genome amplification strategy for forensic genetic analysis using single or few cell equivalents of genomic DNA." *Analytical Biochemistry*. (2005): 246-57. Print.
- [49] Zhang, Lin, Xiangfeng Cui, et al. "Whole genome amplification from a single cell: Implications for genetic analysis." *PNAS*. 89. (1992): 5847-51. Print.
- [50] Kuivaniemi, Helena, Sungpil Yoon, et al. "Primer-Extension Preamplified DNA is a Reliable Template for Genotyping ." *Clinical Chemistry*. 48.9 (2002): 1601-04. Print.
- [51] Smith, Pamela, and Jack Ballantyne. "Simplified Low-Copy-Number DNA Analysis by Post-PCR Purification." *Journal of Forensic Science*. 52.4 (2007): 820-29. Print.
- [52] Forster, Luke, Jim Thomson, and Stefan Kutranov. "Direct comparison of post-28 cycle PCR purification and modified capillary electrophoresis methods with the 34-cycle "low copy number" (LCN) method for analysis of trace forensic samples." *Forensic Science International*. (2008). Print.
- [53] Proff, C., M.A. Rothschild, and P.M. Schneider. "Low volume PCR (LV-PCR) for STR typing of forensic casework samples." *International Congress Series*. (2006): 645-47. Print.
- [54] Gaines, Michelle, Patrick Wojtkiewicz, et al. "Reduced volume PCR amplification reactions using the AmpFISTR Profiler Plus kit." *Journal of Forensic Science*. 47.6 (2002): 1-14. Print.
- [55] Schmidt, Ulrike, Sabine Lutz-Bonegel, et al. "Low-volume amplification on chemically structured chips using the PowerPlex16 DNA amplification kit ." *International Journal of*

- Legal Medicine. (2006): 42-8. Print.
- [56] Horsman, Katie, Joan Bienvenue, et al. "Forensic DNA analysis on microfluidic devices: a review." *Journal of Forensic Science*. 52.4 (2007): 784-99. Print.
- [57] Masseret, Estelle, Marjorie Enquebecq, et al. "A simple and innovative method for species identification of phytoplankton cells on minute quantities of DNA ." *Environmental Microbiology Reports*. 2.6 (2010): 715-9. Print.
- [58] Woide, Daniela, Albert Zink, and Stefan Thalhammer. "Technical Note: PCR Analysis of Minimum Target Amount of Ancient DNA." *American Journal of Physical Anthropology*. 142. (2010): 321-7. Print.
- [59] Babbe, Holger, Axel Roers, et al. "Clonal Expansions of Cd8 T Cells Dominate the T Cell Infiltrate in Active Multiple Sclerosis Lesions as Shown by Micromanipulation and Single Cell Polymerase Chain Reaction." *JEM*. 192.3 (2000): 393-404. Print.
- [60] Kuppers, R., M. Zhao, et al. "Tracing B cell development in human germinal centres by molecular analysis of single cells picked from histological sections." *EMBO Journal*. 12.13 (1993): 4955-67. Print.
- [61] Elliot, K., D.S. Hill, et al. "Use of laser microdissection greatly improves the recovery of DNA from sperm on microscope slides." *International Congress Series*. 1261. (2004): 45-7. Print.
- [62] Sanders, Christine, Nick Sanchez, et al. "Laser Microdissection Separation of Pure Spermatozoa from Epithelial Cells for Short Tandem Repeat Analysis." *Journal of Forensic Science*. 51.4 (2006): 748-57. Print.
- [63] Murray, Caroline, Colin McAlister, and Keith Elliot. "Identification and isolation of male cells using fluorescence in situ hybridisation and laser microdissection, for use in the investigation of sexual assault." *Forensic Science International: Genetics*. (2007). Print.